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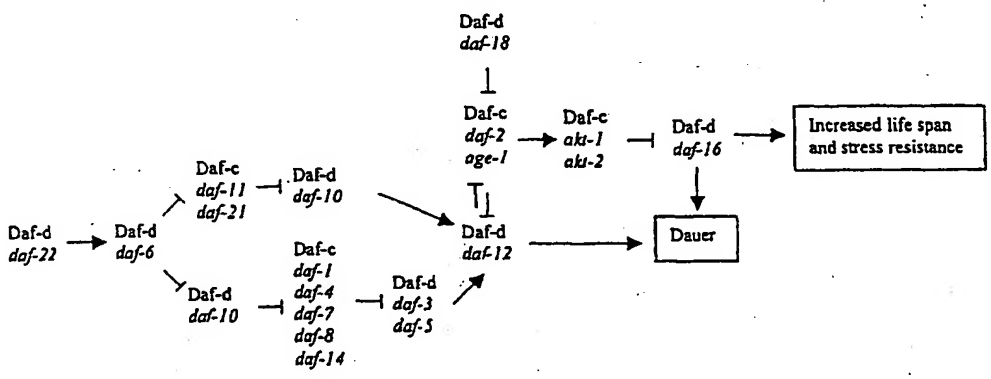
(54) Title: METHOD FOR IDENTIFYING MODULATORS OF DAF-16



(57) Abstract: The invention comprises methods for identifying agents that can activate *C. elegans* DAF-16 and human homologs thereof, or their corresponding genes, whereby cytoprotective effects in cells may be induced. Such cytoprotective effects can result in enhanced environmental stress resistance, increased life span and improved late life vigor without significant inhibition of insulin-signaling pathways. The invention also comprises the therapeutic agents identified and methods of treatment using the agents.

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WO 9851351 A	19-11-1998	AU 7494198 A	08-12-1998
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(54) Title: THERAPIES AND REAGENTS FOR INCREASING STRESS RESISTANCE AND LIFE SPAN

(57) Abstract: The invention comprises methods for identifying agents that can activate *C. elegans* DAF-16 and human homologs thereof, or their corresponding genes, whereby cytoprotective effects in cells may be induced. Such cytoprotective effects can result in enhanced environmental stress resistance, increased life span and improved late life vigor without significant inhibition of insulin-signaling pathways. The invention also comprises the therapeutic agents identified and methods of treatment using the agents.

THERAPIES AND REAGENTS FOR INCREASING STRESS RESISTANCE AND LIFE SPAN

BACKGROUND OF THE INVENTION

The elderly are at greater risk for many chronic diseases, including Alzheimer's Disease (AD), cancer, diabetes and Parkinson's Disease. AD is a progressive neurodegenerative disorder that primarily affects the elderly. AD is the most common form of dementia in persons over the age of 65, with an estimated 7-10% of the population over 65 years old affected, and up to 40% of the population over 80 (McKhann, G. et al. (1984) Neurology 34:939-44; and Evans, D. et al. (1989) JAMA 262:2551-6).

Prevention and treatment of the diseases of the aging will require an understanding of the underlying molecular processes. Unfortunately, research on aging has been hampered by both the technical difficulties of studying life spans in animals, and the inherently complex nature of the process. The complexity of the problem has spawned numerous theories to explain the aging process ranging from genomic instability, shortening telomeres, and oxidative stress (for recent reviews see Johnson, F. et al. (1999) Cell 96:291-302; and Beckman, K. et al. (1998) Physiol. Rev. 78:547-81). This complex evidence has made it difficult to unify aging into a coherent picture.

Recently, the study of aging in model organisms has greatly increased our understanding of this fundamental process. Many of these studies have revealed a link between resistance to environmental stress and increased life span. This link has been well studied in the nematode *Caenorhabditis elegans*. Since mutations in many genes would lead to decreased life span, genetic screens were done for mutations that would result in longer-lived *C. elegans*, the Age phenotype. The first Age mutations identified were in the gene *age-1*. *age-1* mutants live approximately 65% longer than wild type animals (Friedman D. et al. (1988) J. Gerontol. 43:B102-9; and Johnson, T. (1990) Science 249:908-12). Closer inspection of these long-lived mutants revealed that they are more resistant to oxidative (Larsen, P. (1993) PNAS 90:8905-9; and Vanfleteren, J. (1995) FASEB J. 9:1355-61), ultraviolet and thermal stress (Lithgow, G. et al. (1995) PNAS 92:7540-4; and Murakami, S. et al. (1996) Genetics 143:1207). This link has been confirmed in several other model organisms. In the yeast *S. cerevisiae*, Kennedy et al. (1995) Cell 80:485-96, used this connection to perform a genetic screen for starvation-

resistant yeast. Four genes were isolated that were more resistant to starvation, and increased life span by 20-55%. In *Drosophila melanogaster* direct screens for long-lived mutants led to the identification of the *methuselah* gene in which mutations increased mean life span ~35%. Along with increasing life span, *methuselah* mutants also possessed an increased resistance to a variety of environmental stresses, including oxidative stress, starvation and high temperature (Lin, Y. et al. (1998) Science 282:943-6).

Genetic and molecular studies of aging in *C. elegans* have converged on another genetic pathway specifying the decision to form dauer larvae. For example, it is now believed that *age-1* is allelic to a gene in the dauer pathway (*daf* genes) called *daf-23*. Mutations in another dauer gene, *daf-2*, also increases life span up to 100% (Kenyon, C. et al. (1993) Nature 366:461-4). Analysis of these genes have revealed a pathway that *C. elegans* uses to sense its environment and adapt its metabolism and life cycle. This pathway includes *daf-2* and *age-1*. Molecular analysis of this pathway has begun to reveal how genetic and biochemical alterations can lead to increased stress resistance and life span. Under culture conditions rich in food, *C. elegans* hermaphrodites continue through four molts to become fertile adults that typically live 3 weeks at 20°C. Under conditions of low food and high population density, a decision is made at the second larval stage to suspend development and enter the dauer stage. The dauer stage is a specialized dispersal form that can live many months (five to eight times longer than non-dauers). Dauer larvae upregulate several stress-resistance enzymes and other stress proteins, such as superoxide dismutase (Anderson, G. (1982) Can. J. Zool. 60:288-291; and Larsen, P. (1993) PNAS 90:8905-9), catalase (Vanfleteren, J. et al. (1995) FASEB J. 9:1355-61) and HSP90 (Dalley, B. et al. (1992) Dev. Biol. 151:80-90). These stress proteins may aid in survival of prolonged dauer states.

Genetic dissection of the dauer pathway has identified a complex set of interacting genes (see Figure 1) (for review see Riddle and Albert (1998) Genetics 150:129-55). These genes can be put in two categories based on their mutant phenotypes, dauer defective (Daf-d) and dauer constitutive (Daf-c). An example of dauer-defective mutations are those that disrupt the ability of the animal to sense its environment, and hence interfere with the ability to respond to crowded conditions. This class of Daf-d mutations also often show defects in chemotaxis or osmotic avoidance, and often have

abnormal morphogenesis of sensory neurons. Since a constant signal of abundant food and lack of overcrowding are required to inhibit dauer formation, mutations in the dauer pathway frequently result in a constitutive dauer phenotype. Daf-c mutants will enter the dauer pathway even when conditions are not appropriate, and include *daf-1*, *daf-2*, *daf-4*, *daf-7*, *daf-8*, *daf-11*, *daf-14*, *daf-21* and *age-1*. All the mutant alleles of *daf-1, daf-4, daf-7, daf-8, daf-11, daf-14* and *daf-21* are temperature sensitive (ts), suggesting that disruption of this arm of the pathway is inherently ts. *daf-2* and *age-1* are associated with another arm of the pathway, and strong loss-of-function mutations in these genes are either lethal or non-conditional dauer constitutive (Riddle et al. (1997), *supra*).

The extension of life span is not simply inappropriate activation of the dauer pathway. Shifting conditional mutations in most Daf-c genes to restrictive temperature late in life has no effect on life span. Also, mutations in *daf-2* and *age-1* will increase life span at temperatures that normally would not induce dauer formation (Kenyon, C. et al. (1993) Nature 366:461-4; and Larsen, P. et al. (1995) Genetics 139:1567-83). Additionally, the activities of *daf-2* are not limited to the dauer decision. Studies with temperature-sensitive alleles of *daf-2* reveal a role for an insulin-like signaling pathway throughout nematode life. The strong *daf-2*(ts) allele *(e979)* is 100% embryonic lethal in homozygous animals at the restrictive temperature, implicating a role for *daf-2* during early development (Gems, D. et al. (1998) Genetics 150:129-55). Similarly, when strong *daf-2(ts)* alleles are shifted to restrictive temperature as adults, the animals adopt a series of characteristic phenotypes. The shifted animals become egg-laying defective, have reduced movement and exhibit body shrinkage, all of which suggest a role for *daf-2* in adult nematodes (Gems et al. (1998), *supra*). *age-1* (hx546) mutants have essentially normal growth and development (Friedman, D. et al. (1988) J. Gerontol. 43:B102-9) but show a modest effect on fertility (Lithgow, G. et al. (1995) PNAS 92:7540-4). The requirement for *daf-2* throughout nematode life suggests that this arm of the dauer pathway functions to promote growth and regulate metabolism throughout the life span, while other arms of the pathway are specific for entry into the dauer stage.

The function of *daf-2* and *age-1* as metabolic regulators is consistent with their molecular identity. *age-1* is predicted to encode a phosphatidyl-inositol 3 kinase (PI3K), similar to the p110alpha that plays a role in mammalian insulin signaling (Morris, J. et al.

(1996) Nature 382:536-9). *daf-2* encodes an insulin-like growth factor receptor (Kimura, K. et al. (1997) Science 277:942-6). Other players in this pathway have recently been identified, and include; *akt-1*, *akt-2*, *daf-18* (homologous to mammalian PTEN) and *daf-16* (homologous to mammalian AFX, FKHR or FKHRLI). Screens for suppressors of the *age-1* dauer-constitutive phenotype isolated a gain-of-function allele of *akt-1*. Gain-of-function alleles or increased dosage of wild-type *akt-1* suppress the Daf-c phenotype of *age-1* mutants. AKT-1 is homologous to mammalian AKT/PKB (Protein Kinase B) (Paradis, S. et al. (1998) Genes Dev. 12:2488-98). *akt-2* was identified in the *C. elegans* genomic sequence by homology to *akt-1*, and they seem to be at least partially redundant. Inhibition of either *akt-1* or *akt-2* alone is not sufficient to induce a Daf-c phenotype, while inhibition of both genes results in a constitutive dauer formation (Paradis, S. et al. (1998), *supra*). This redundancy may explain why these genes were not identified in previous dauer screens. AKT-1/2 may be the major outputs of PI3K (AGE-1) signaling in worms, while DAF-2 is likely to have other outputs (Paradis, S. et al. (1998), *supra*.

Genetic and biochemical studies now suggest that the *daf-16* gene is central to the increase in longevity associated with *daf-2* and *age-1* mutations. The increase in life span conferred by *age-1* and *daf-2* mutations can be fully suppressed by loss of function mutations in *daf-16*. *daf-16* mutants were originally identified as Daf-d, with no other obvious phenotypes. *daf-16* encodes a putative transcription factor with a forkhead, or winged helix, DNA binding domain (Lin, K. et al. (1997) Science 278:1319-22; and Ogg, S. et al. (1997) Nature 389:994-9). Three human homologues to DAF-16 have been identified, AFX, FKHR and FKHRL1. FKHR had previously been identified as playing a role in alveolar rhabdomyosarcoma, an aggressive pediatric soft tissue tumor. Analysis of tumors detected a translocation that fuses the DNA binding domain of PAX3 or PAX7 to the carboxy-terminus of FKHR (Davis, R. et al. (1994) Cancer Res. 54:2869-72; and Galili, N. et al. (1993) Nat. Genet. 5:230-5). The C-terminus of FKHR contains a potent transcriptional activation domain, which may lead to strong unregulated activation of PAX3 or PAX7 targets (Sublett, J. et al. (1995) Oncogene 11:545-52). AFX was also identified by its role as a tumorogenic translocation (Borkhardt, A. et al. (1997) Oncogene 14:195-202). FKHRL1 was identified by homology to FKHR and AFX (Anderson, M. et al. (1998) Genomics 47:187-99).

The homology of FKHR, FKHRL1 and AFX to DAF-16 suggests that the endogenous proteins may function in insulin signaling in mammals in a conserved pathway similar to the one identified in *C. elegans*. Biochemical studies on the mammalian homologues of DAF-16 suggest how growth factor signaling regulates the activity of this family of transcription factors. In mammalian cells, growth factor signaling activates the serine threonine kinase AKT, which leads to the phosphorylation of three sites on FKHRL1. Phosphorylation of FKHRL1 inhibits its entry into the nucleus preventing it from activating transcription of target genes (Brunet, A. et al. (1999) Cell 96:857-68). AFX is also repressed by AKT through insulin signaling. This biochemical model of inhibition of mammalian DAF-16 homologs by growth factor signaling is strikingly similar to the genetic pathway in *C. elegans*. Epistasis analysis of the dauer pathway suggests that upstream signaling molecules function to negatively regulate *daf-16* (see Figure 1). It has been suggested that DAF-16 in *C. elegans* promotes longevity in that organism by repressing metabolic genes that mediate energy usage. This results in a shift to an energy-conservation state in which the nematode increases fat storage deposits. The conservation of the genetic pathway from nematodes to mammals suggests that they may affect similar processes. However, it is not to be expected that homologous target genes should be the sites of DAF-16 binding in nematodes and in mammals.

There have been a number of suggestions in the literature that unregulated activity of mammalian DAF-16 homologs may lead to a number of disease states. See, Ruvkun et al., WO 98/51351. For example, Ruvkun et al have suggested that it is the dysregulation of DAF-16 homologs -- *i.e.*, inappropriately high levels of DAF-16 homolog activity even in the presence of ample glucose -- that leads to a number of the metabolic defects seen in individuals with type I and II diabetes. Such individuals have a decline in insulin-dependent signaling, and so presumably have a higher level of DAF-16 homolog activity in the presence of glucose than in wild type individuals. It has therefore been suggested that compounds that can downregulate the activity of DAF-16 homologs may be useful as therapeutic agents for the treatment of individuals with diabetes and other conditions that result from a decrease in the insulin signaling pathway.

SUMMARY OF THE INVENTION

It would be expected from the prior art that increasing the activity or dosage of DAF-16 homologs would lead to detrimental effects on cells. Indeed, the Ruvkun et al. patent (WO 98/51351) describes methods and reagents for lowering the level of DAF-16 homolog activity by either up-regulating the activity of the proteins that negatively regulate DAF-16, or down-regulating DAF-16 itself. However, the present invention unexpectedly reveals novel beneficial functions of DAF-16. In particular, the present invention reveals for the first time that increased dosage of *daf-16* greatly increases the resistance to heat. In addition, the present invention shows for the first time that increased dosage of *daf-16* increases resistance to ultraviolet light. Third, the present invention shows that *daf-16* will potentiate the increase in life span induced by a mild heat shock. Fourth, the present invention demonstrates for the first time that increased activity of *daf-16* leads to increased protein levels of some members of the stress response pathway. Fifth, the present invention demonstrates for the first time that increased *daf-16* dosage will increase resistance to oxidative stress. These cytoprotective effects are not accompanied by phenotypes resulting from defects in the insulin-signaling pathway, *e.g.*, constitutive dauer formation is not observed. Thus, the present invention provides for the first time evidence that increasing the activity of DAF-16 homologs may have beneficial therapeutic effects in mammals (e.g., humans) without accompanying detrimental effects on insulin-dependent signaling.

The present invention includes methods and reagents that increase the dosage of DAF-16 homologs in human cells. In some embodiments, the invention provides isolated DNA encoding DAF-16 or DAF-16 homologs in a vector that is capable of directing the expression of DAF-16 or DAF-16 homologs in human cells. The isolated DNA in such a vector is operatively linked to a promoter that can direct the expression of the polypeptide either in all tissues in the body, or within certain subsets of tissues, or within single cell types.

The invention also includes methods for identifying agents that increase the activity of DAF-16 homologs in mammalian (e.g., humans) cells. The invention further includes methods for administering such reagents to humans.

The methods of identifying agents that enhance the activity of DAF-16 or a DAF-16 human homolog have a number of embodiments. In one embodiment, the method comprises (a) contacting DAF-16 or a DAF-16 human homolog with candidate agents under assay conditions for DAF-16 activity or DAF-16 human homolog activity; and (b) identifying those agents that enhance the activity of the DAF-16 or DAF-16 human homolog. In another embodiment, the method comprises (a) contacting *daf-16* or a *daf-16* human homolog with candidate agents under conditions permitting expression; (b) conducting an assay for DAF-16 activity or DAF-16 human homolog activity; and (c) identifying those agents that enhance the activity of the DAF-16 or DAF-16 human homolog.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the *C. elegans daf-16* dauer pathway.

Figure 2 demonstrates the stress resistance of ExR13H8 strains. Figure 2a illustrates the fraction of *C. elegans* surviving for sterile (Str) and fertile (Fer) animals derived from two independent lines, ExR13H8(1) and ExR13H8(2) at 37.5°C for 2 hrs. Figure 2b illustrates the fraction of *C. elegans* surviving for *age-1(hx-546)*;ExpRF4 and a sterile ExR13H8 strain at 37.5°C for 2.5 hrs. Figure 2c illustrates that N2 (wild-type) nematodes with R13H8 extra-chromosomal arrays are more resistant to UV ($P<0.0002$). Figure 2d illustrates that sterile animals derived from *age-1(hx-546)*;ExR13H8 strains are more resistant to UV ($P<0.0001$). Figure 2e illustrates that N2;ExR13H8(Str) animals are more resistant to peroxide treatment than N2;ExpRF4 or *age-1(hx-546)*;ExpRF4 ($P<0.0001$).

Figure 3 demonstrates that heat treatment extends the life span of ExR13H8 strains. N2;ExpRF4 HS (heat shocked) and N2;ExR13H8 (1) HS curves are significantly different ($P<0.0001$). Data shown is from one representative experiment, and has been replicated.

Figure 4 illustrates the expression of DAF::GFP in nematodes under various conditions. Figure 4a is a DIC (differential interference contrast) image of ExDAF-16::GFP strain. Figure 4b (a fluorescence microscopy image, FMI) demonstrate that DAF-16::GFP is widely expressed and largely cytoplasmic. Note the lack of expression in

germ line (g) of inset. Figure 4c is a DIC image of ExDAF-16GFP strain after one hour 34°C heat treatment. Figure 4d (a FMI) illustrates localization of DAF-16::GFP to the nucleus after heat treatment (arrows). Figure 5 illustrates the stress resistance of ExDAF-16::GFP strains. Figure 5a shows that N2 nematodes with extrachromosomal arrays of DAF-16::GFP are more thermotolerant. Error bars represent standard error. Figure 5b demonstrates that N2;ExDAF-16::GFP animals are more UV resistant than N2;ExpRF4. Curves are significantly different ($P<0.0001$). Figure 5c illustrates the life spans of N2;ExpRF4 and N2;ExDAF-16::GFP strains at 20°C. ExDAF-16::GFP animals live longer than N2;ExpRF4 animals ($P<0.0003$). Life spans were determined in parallel for each strain. Date are from one representative experiment and have been replicated.

Figure 6 illustrates HSP70 levels in aging wild-type *C. elegans* and *daf-16(mgDf50)* animals. Figure 6a is a Western blot of HSP70 levels from five adults of the indicated age. Figure 6b is the optical density corresponding to each of the Figure 6a lanes. Figure 6c illustrates the life span of wild-type and *daf-16(mgDf50)* at 25°C.

Figure 7 contains Western analyses for HSP70, HSP90 and ubiquitin in a number of strains. Figure 7a shows unchanged HSP90 levels in wild-type (N2), *age-1(hx-546)*, N2;ExpRF4, and N2;ExR13H8. Figure 7b shows that HSP70 levels are higher in sterile ExR13H8 animals. Figure 7c shows ubiquitin levels in ExR13H8 strains. Arrows indicate prominent bands. Figure 7d illustrates HSP70 levels in N2;ExpRF4 and N2;ExDAF-16::GFP strains. Figure 7e shows ubiquitin levels in N2;ExpRF4 and N2;ExDAF-16GFP strains.

Figure 8 illustrates routes of DAF-16 activation. Figure 8a depicts insulin-like signaling that activates AKT-1/2 which phosphorylates DAF-16 enhancing binding to a cytoplasmic retention factor, preventing activation of genes involved in increased stress resistance and life span. Figure 8b illustrates low insulin signaling which results in dephosphorylation of DAF-16, loss of binding to cytoplasmic retention factor and nuclear localization. Figure 8c shows a possible mechanism for heat activation of DAF-16-like molecules. This model is only one of several possible mechanisms for activation of DAF-16-like proteins.

Figure 9 depicts the insulin signaling pathways of mammals.

Figure 10 illustrates a construct useful in identifying agents that up-regulate transcription of DAF-16 human homologs.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention comprises methods of identifying agents that can increase the activity of DAF-16 or mammalian (e.g., human) homologs thereof. These agents can be used therapeutically to enhance cytoprotective activities in cells. Discussed in detail herein are methods and assays for screening candidate molecules to identify those agents that can have such therapeutic effects or which can be the subject of further drug discovery methods known in the art to obtain compounds that have therapeutic value in mammals, in particular humans.

This invention is also directed to the agents obtained by the assays described therein, and to the therapeutic treatment of mammals (e.g., humans) with such agents to increase life span, improve late life vigor, enhance resistance to environmental stress, and otherwise protect cells from damage. It is also foreseen that the agents obtained using the subject assay methods may be useful in the induction of apoptosis of tumor cells.

In another embodiment, the subject invention concerns a method for increasing the dosage of one or more DAF-16 homologs in mammalian (e.g., human) cells via gene therapy. Each of these embodiments is described in detail below.

The foregoing agent screening assays, agents and methods of treatment are based on extensive experiments conducted in *C. elegans* that reveal that activation of DAF-16 can result in cytoprotective effects without the adverse effects associated with the perturbation of insulin signaling pathways observed in some diabetics. These experimental results are discussed in detail below and are presented in the Examples.

A number of terms used herein are defined as follows:

"Candidate agents" or "candidate molecules" means agents or molecules that can be tested in screening assays for suitability as agents to enhance DAF-16 or DAF-16 human homolog activity. Typically, candidate agents are small molecules, peptides, oligonucleotides and/or derivatives thereof, or other compounds known to be useful as screening candidates in the drug discovery field.

"Cytoprotective activity" means activity that enhances a cell's resistance to environmental stress such as elevated temperature, oxidative stress or ultraviolet irradiation. Through such cytoprotective activity, a cell or organism may exhibit improved UV resistance, improved thermotolerance, improved oxidative stress resistance, improved life span and/or improved late life vigor. The cytoprotective activity may be effected through activation of HSP70, HSP90, catalase and/or superoxide dismutase.

"Functional *C. elegans* DAF-16" refers to a *C. elegans* DAF-16 that, when activated, performs its wild-type function of inducing cytoprotective activity.

"Human homolog" of DAF-16 refers to the products of the human genes FKHR, FKHRL1, AFX or to a human polypeptide containing a region that has at least 50% identity with the winged helix domain of *C. elegans* DAF-16. The winged helix domain of DAF-16 is the putative DNA binding region and is of the amino acid sequence: KKTTTRRNAWGNMSYAELITTAIMASPEKRLTLAQVYEWMVQNVPYFRDKGDS NSSAGWKNSIRHNLSLHSRFMRIQNEGAGKSSWWVINPDAKPGR. With increasing preference the human homolog has at least 60%, 70% 80%, 90% and 95% identity to this region. The human homolog also has a function similar or analogous to that performed by DAF-16. Preferably retains at least 50%, 60%, 70%, 80%, 90%, or 95% of the desired DAF-16 activity.

"Impaired insulin signaling" or "perturbation in insulin signaling" in *C. elegans* refers to genetic or other alterations that lead to reduced activity in the insulin-like signaling pathway in nematodes. These alterations include, but are not limited to, inhibition of expression or activity of a set of genes defined as homologous to genes involved in insulin-like signaling in mammals. Examples of such genes are *daf-2, age-1, pdk-1, akt-1, akt-2, daf-18,* and *daf-16.* Impaired insulin signaling will result from null, loss of function or reduction of function alleles from these as well as other genes in this pathway.

"Impaired insulin signaling" or "perturbation in insulin signaling" in humans refers to alterations that lead to reduced activity of genes or gene products of the insulin signaling pathway in mammals. These alterations include genes and gene products that are generally recognized as being downstream of activation of Insulin receptor or IGF-1 receptor.

"Improved target protein activation" or "enhanced target protein activation" means increased target protein gene transcription, translation and/or target protein activation.

"Increased activity" or "enhanced activity" of DAF-16 or DAF-16 homologs refers to increased *daf-16* or *daf-16* human homolog transcription or translation, increased DAF-16 or DAF-16 homolog activation and/or increased target protein activation.

"Operatively linked" refers to the conjugation of a polypeptide encoding oligonucleotide to a regulatory region or leader containing binding sites or response elements in such a manner to permit the transcription and/or translation of the oligonucleotide. Regulatory or leader regions and methods for assuring proper linkage to oligonucleotides are known in the art.

"Target protein" of DAF-16 or a DAF-16 human homolog refers to any protein that DAF-16 or DAF-16 homolog either binds to directly to activate or whose transcription or translation is activated by binding of DAF-16 or a DAF-16 homolog to the regulatory region of the gene or the mRNA encoding the protein. Target proteins can include, but are not limited to, HSP70, HSP90, catalase, ubiquitin and/or superoxide dismutase.

Discussion of *C. elegans* Experimental Results and Relevance to Medical Applications in Humans

In *C. elegans,* the activity of DAF-16 is central to the life extension seen in the Daf-c mutants *daf-2* and *age-1* (Gottlieb, S. et al. (1994) Genetics 137:107-20; and Ogg, S. et al. (1997) Nature 389:994-9). In this invention it has been shown that: (1) increasing the dosage of *daf-16* greatly increases the stress resistance of transgenic nematodes; (2) increasing the copy number of *daf-16* leads to a large increase in the life span of animals subjected to repeated small heat shocks; and (3) the increase in resistance to heat and UV may be due in part to increased levels of HSP70.

The genetics of aging in *C. elegans* has uncovered a molecular pathway homologous to mammalian insulin signaling. In nematodes, reduced insulin signaling leads to increased stress resistance and life span (Lithgow, G. et al. (1995) PNAS 92:7540-4; Murakami, S. et al. (1996) Genetics 143:1207-18; Kimura, K. et al. (1997) Science 277:942-6; Morris, J. et al. (1996) Nature 382:536-9; and Tissenbaum, H. et al. (1998) Genetics 148:703-17). This suggests a model in which a limited food supply results in

reduced insulin/IGF-1 levels and functions to prepare the animal for an endurance lifestyle, whereas abundant food and high insulin levels trigger a reproductive life style. The choice between a reproductive versus endurance life style affects the life span and overall health of the animal, and is regulated by the DAF-16 family of transcription factors.

The choice of reproductive versus endurance life styles is very apparent in *C. elegans*. In *C. elegans*, high food concentrations and low population density signals the reproductive life style, whereas low food availability and high population density triggers the dauer (endurance) stage. This life style switch is found in many species. For example, Mediterranean fruit flies exhibit "dual modes of aging" characterized by a waiting mode and a reproductive mode. The choice of mode is dictated, at least in part, by diet. Flies fed only sugar exist in a waiting mode, don't reproduce, and have increased life spans compared to animals fed a full diet (Carey, J. et al. (1998) Science 281:996-8). Flies fed a full diet of sugar and protein (protein is limiting in the wild) reproduce rapidly and have shorter life spans (Carey, J. et al. (1998), *supra*). This is similar to the effect of caloric restriction (CR) on increasing longevity and stress resistance in rodents (for review see (Masoro, E. (1998) Aging (Milano) 10:173-4). Animals fed a very low caloric diet have low levels of circulating IGF-1 (Dunn, S. et al. (1997) Cancer Res. 57:4667-72). The low levels of IGF-1 seem to be critical to the resistance to tumor formation in these animals (Mukherjee, P. et al. (1999) J. Natl. Cancer Inst. 91:512-23; and Kritchevsky, D. (1997) Breast Cancer Res. Treat. 46:161-7). CR female rodents become sterile, show greater stress resistance and increased life span. Male rodents show similar increases in stress resistance and life span, but continue to make sperm (see Masoro, E. (1998), *supra*).

Genetic analysis in *C. elegans* has dissected how nematodes make the choice between the reproductive versus endurance life style. The dauer pathway represents a complex series of interacting genes that *C. elegans* uses to sense and respond to environmental conditions (see Figure 1). Mutations in one arm of this pathway, homologous to mammalian insulin signaling, function to increase life span and stress resistance. Genetic and biochemical analysis has identified the genes *daf-2*, *age-1*, *akt-1/2*, *daf-18* and *daf-16* as key players in this pathway. DAF-16 is a transcription factor

that is activated by low levels of insulin signaling and is responsible for both the beneficial and harmful effects of reduced insulin signaling.

According to the present invention (see Examples), it is seen that *C. elegans daf-16* is required for the increased stress resistance and longevity phenotypes associated with reduced insulin signaling. Increased dosage of *daf-16* containing cosmid R13H8 dramatically increases resistance to heat and ultraviolet stress. Since the transgenes are maintained as extrachromosomal arrays, the animals are likely to be mosaic, and some animals are expected to be more affected then others. Because *daf-2(lf)* (loss of function) mutations exhibit a sterile phenotype, the inventors predicted that the sterile animals derived from ExR13H8 strains would be the most strongly affected. Consistent with this idea, it was found that the sterile animals were the most stress resistant. The majority of animals remain fertile, yet under standard culture conditions, the animals are kept well fed and in uncrowded conditions, and it is likely that endogenous AKT activity is inhibiting the activity of excess DAF-16. This is consistent with the observation that the frequency of steriles is increased in an *age-1(rf)* (reduced function) background, where PI3K activity is reduced (data not shown).

The increase in thermotolerance has been found to be due to *daf-16*. Microinjection of dsRNA specific to *daf-16a* coding region eliminates the increased thermotolerance of these animals. This experiment rules out the possibility that other genes on the cosmid contribute to the effect. Another possibility is that the Itt (increased thermotolerance) phenotype is due to increased amount of genomic sequences on the transgene. For example, extra copies of promoter regions could titrate out a limiting transcription factor for other genes. This model is unlikely, however, since RNAi is thought to inhibit activity of the target genes (see Tabara, H. et al. (1998) Science 282:430-1). Therefore, the increase in thermotolerance is almost certainly due to increased expression of *daf-16*. Since the effect of *daf-16* RNAi on the germ line was weak, other models for this effect cannot be totally ruled out.

An increased copy number of cosmid R13H8 was tested to see if it would increase mean life span of transgenic nematodes relative to controls. Unselected transgenics were first tested, with no bias towards fertility or sterility. Under constant temperature, no increase in life span was observed between N2;ExpRF4 and unselected N2;ExR13H8

strains (see Table 4). Given the strong thermotolerant phenotype of sterile N2;ExR13H8 animals, the life span of sterile animals and fertile animals derived from N2;ExR13H8 was tested versus controls. No significant extension of mean life span was observed (data not shown). One explanation for the lack of life span extension concerns the lack of transgene expression in the germ line of *C. elegans*. The germ cells of *C. elegans* exert a negative influence on life span, and this effect operates through a DAF-16 pathway (Hsin et al. (1999) Nature 399:362-366). In long-lived mutants, DAF-16 activity would be up-regulated in the germ line, while this would not occur in transgenics.

Surprisingly, it was seen that when ExR13H8 strains were maintained in a fluctuating temperature environment an increase in life span was observed. This phenomenon was tested systematically by administering a mild repeated heat shock. This heat treatment led to increases in both mean and maximum life spans. The increases in life span cannot be due to simple thermo-protection since the animals lived considerably longer than non-heat shocked animals. This result is surprising, since in mammalian cells heat stress activates AKT (Konishi, H. et al. (1997) FEBS Lett. 410:493-8; Matsuzaki, H. et al. (1996) FEBS Lett. 396:305-8; and Shaw, M. et al. (1998) Biochem. J. 336:241-6). Perhaps in *C. elegans* the effect is reversed, or some other mechanism leads to activation, such as dissociation of DAF-16 from cytoplasmic 14-3-3 proteins or activation of a protein phosphatase.

One result of increased dosage of *daf-16* is increased expression of HSP70. Levels of HSP70 protein were much higher in sterile ExR13H8 animals than in wild type. This is consistent with an increase in HSP70 protein levels seen in *age-1(hx546)* versus wild type. Increased dosage of HSP70 has been reported to increase life span of *Drosophila* (Tatar, M. et al. (1997) Nature 390:30). No increase in HSP90 protein levels was seen, even though dauer larvae have large increases in HSP90 mRNA (Dalley, B. et al. (1992) Dev. Biol. 151:80-90). The large increase in HSP70 levels may explain the strong thermotolerance in these animals. Yet, it is likely that increasing the dosage and activity of DAF-16 activates many other stress response genes, such as catalase (Taub, et al. (1999) Nature 399:162-166).

Increased dosage of *daf-16* also resulted in increased expression of ubiquitin in sterile ExR13H8 animals. Ubiquitin is used in the degradation of short-lived or mis-folded

proteins (for review see Hershko, A. et al. (1998) Annu. Rev. Biochem. 67:425-79). Ubiquitin is essential for the stress response in yeast (Finley, D. et al. (1987) Cell 48:1035-46), and its expression is regulated by insulin (Larbaud, D. et al. (1996) Am. J. Physiol. 271:E505-12). The presence of insulin response elements in the regulatory sequences of the human ubiquitin B gene (Baker, R. et al. (1987) Nucl. Acids Res. 15:443-63) suggests that it may be regulated, in part, by the DAF-16 homologs. Since the antibody recognized both ubiquitinated proteins as well as ubiquitin itself, some of the increased levels represent tagged proteins.

The increase in HSP70 and ubiquitin levels may be due to increased levels of DAF-16 protein, however it is possible that excessive DAF-16 forms aggregates and thereby stimulates a stress response. Yet, given the role for *daf-16* in stress resistance in long-lived nematodes and its central role in insulin signaling, it is likely that the gains in stress resistance are due to increased levels and activation of DAF-16.

As is discussed above, it was found that under constant temperature, there was little or no increase in life span in ExR13H8 animals. However, if the animals were subjected to a fluctuating environment, increases in life span were noted. To examine this effect more closely, a DAF-16:GFP fusion protein was used to visualize the sub-cellular localization of DAF-16. Under standard culture conditions most of the DAF::GFP fusion protein is not localized (see Figure 5b). Typical culture conditions provide the animals with abundant food in an uncrowded environment. Under such ideal conditions, insulin-like signaling is probably sufficient to exclude most DAF-16::GFP from the nucleus, even when present in increased dosage. Yet, a mild heat shock led to dramatic nuclear localization of the DAF-16::GFP fusion protein and increased life span. This indicates multiple routes for activation of DAF-16, using either hormonal regulation by insulin-like signaling, or by direct external stimuli. A possible model for this activation is shown in Figure 8. FKHR, a mammalian DAF-16 homolog, is phosphorylated by AKT, which induces FKHR binding to a 14-3-3 protein and its retention in the cytoplasm (Brunet, A. et al. (1999) Cell 96:L857-68). DAF-16 may be excluded from the nucleus by a similar mechanism. A heat treatment may transiently denature the association of DAF-16 from its cytoplasmic partner and allow for nuclear localization. Alternatively, heat treatment may deactivate AKT-1/2 or activate a phosphatase, which changes the phosphorylation state of

DAF-16. DAF-16 may be activated by temperature as a direct method to modulate stress resistance and life span in nematodes based on environmental signals.

Consistent with increased dosage of the cosmid R13H8, increased dosage of DAF-16::GFP fusion protein increased stress resistance and life span (Figure 5).

The DAF-16 family of transcription factors may act to direct a set of responses to low food availability, and prepare the organism for stress. Inappropriate activation of DAF-16 in the presence of abundant food, as occurs in *age-1* and *daf-2* mutations appears to give rise to several beneficial effects. Genetic and biochemical data from invertebrates and vertebrates suggest how DAF-16 is activated. Insulin signaling leads to activation of the serine/threonine kinase AKT. AKT directly phosphorylates the DAF-16 homologue FKHRL1, which prevents it's entry into the nucleus, and activation of target genes (see Figure 9) (Brunet, A. et al. (1999) Cell 96:857-68). Target genes of the DAF-16 family are likely to be the mediators of the endurance pathway. For example, withdrawal of growth factors increases apoptosis in cell culture, and this is believed to be important in tumor resistance in caloric restricted animals (Mukherjee et al. (1999), *supra*; and Kritchevsky, D. (1997) Breast Cancer Res. Treat. 46:161-7). One of the identified targets of FKHRL1 is the apoptosis promoting gene for the Fas ligand. Other candidate genes for activation by DAF-16 are catalase (Taub, et al. (1999), *supra*), superoxide dismutase, HSP70 and ubiquitin. DAF-16 may regulate the expression of *sod-3* (a superoxide dismutase) (Furuyama, T. (2000) Biochem. 349:629-634). AFX transcriptionally activates p27kip1, a cell cycle regulatory protein (Medema R. (2000) Nature 404:782-7).

Increased expression of stress response proteins has proven to be cytoprotective in many cases and have tremendous medical applications (for review see Morimoto et al. (1998) Nat. Biotechnol. 16:833-8). Over-expression of HSP70 in myogenic cells will protect them from ischemic stress and damage by reperfusion (Mestril, R. et al. (1994) J. Clin. Invest. 93:759-67). Furthermore, transgenic mice over expressing HSP70 incur less myocardial damage from ischemia-reperfusion (Marber, M. et al. (1995) J. Clin. Invest. 95:1446-56; and Plumier, J. et al. (1995) J. Clin. Invest. 95:1854-60). Over-expression of antioxidant enzymes such as superoxide dismutase and catalase also have cytoprotective abilities and can extend life span of *Drosophila* (Orr, W. (1994) Science 263:1128-30; and Parkes, T. et al. (1998) Nat. Genet. 19:171-4). The DAF-16 family of transcription factors

appear to be "master regulators" of a wide variety of stress response enzymes. In this invention it has been shown that activation of DAF-16 is cytoprotective in *C. elegans* and protects the cells against both thermal, ultraviolet radiation and oxidative damage. The up-regulation or activation of DAF-16 molecules is likely to result in substantial medical benefits.

Methods of Identifying Agents that Enhance DAF-16 Activity

The results described herein reveal for the first time that increased activity of DAF-16 in nematode cells leads to an increase in cytoprotective activity. These results enable the development of a number of screening systems for identifying therapeutic compounds that increase the activity of DAF-16 homologs in human cells. Such compounds will be useful for increasing the cytoprotective abilities of cells in particular, and potentially for increasing individual longevity and late life vigor in general. This section provides a description of several of the possible screens included within the scope of this invention.

Many methods are known in the art of drug discovery for obtaining compounds that can be screened for a desirable activity. The compounds can be obtained from natural sources -- *e.g.*, plant extracts, fungal extracts etc. -- or they can be chemically synthesized. For example, many techniques are known in the art for synthesizing compounds from a variety of precursors in such a way that the linkage of the individual precursors is randomized or semi-randomized. This leads to a library of randomized or semi-randomized candidate molecules that can then be screened for a particular activity. Once a member of the library with the desired activity is isolated, that member can be further manipulated in an attempt to further increase its activity in the screening assay.

In one embodiment, the subject invention comprises a method of identifying an agent that enhances the activity of DAF-16 or a DAF-16 human homolog, comprising (a) contacting DAF-16 or a DAF-16 human homolog with candidate agents under assay conditions for DAF-16 activity or DAF-16 human homolog activity; and (b) identifying those agents that enhance the activity of the DAF-16 or DAF-16 human homolog. A variation on this method comprises (a) contacting *daf-16* or a *daf-16* human homolog with candidate agents under conditions permitting expression; (b) conducting an assay for DAF-16 activity or DAF-16 human homolog activity; and (c) identifying those agents that

enhance the activity of the DAF-16 or DAF-16 human homolog. The improved DAF-16 or DAF-16 human homolog activity can arise from improved transcription, improved translation, activation (i.e., dephosphorylation) of the DAF-16 itself or a human homolog thereof, or activation of DAF-16 or homolog target proteins.

In another embodiment, the invention includes a method for identifying an agent that increases the activity of DAF-16 in *C. elegans* cells, whereby cytoprotective activity in the cells may be enhanced, comprising (a) contacting candidate molecules with a *C. elegans* strain comprising a wild type *daf-16* locus; (b) screening for animals that exhibit enhanced DAF-16 activity or cytoprotective activity; and (c) identifying the agent that enhanced the DAF-16 activity or cytoprotective activity. Detailed methods for performing this screen are provided herein.

In another embodiment, the invention provides a method for identifying an agent that increases the activity of a DAF-16 human homolog, whereby cytoprotective activity in host or human cells may be enhanced, comprising (a) contacting candidate molecules with a *C. elegans* strain comprising a *daf-16* gene that is mutated or deleted whereby functional *C. elegans* DAF-16 is not produced, and a *daf-16* human homolog gene; (b) screening for animals that exhibit enhanced DAF-16 human homolog activity or cytoprotective activity; and (c) identifying the agent that enhanced the DAF-16 human homolog activity or cytoprotective activity. The *daf-16* human homolog can be present as a transgene on an extrachromosomal array.

The foregoing embodiment operates on the reasonable presumption that a human DAF-16 homolog can induce the dauer state in *C. elegans* or otherwise induce cytoprotective effects *in C. elegans*. It has been found that human FKHRL1 can be phosphorylated and inactivated by *C. elegans* AKT-1/2.

The subject invention also includes a method for identifying an agent that increases the activity of DAF-16 in *C. elegans* cells, whereby cytoprotective activity in the cells may be enhanced, comprising (a) contacting candidate molecules with a *C. elegans* strain having a gain-of-function *daf-2*, *age-1* or *akt-1/2* mutation, whereby DAF-16 is inactivated even in the absence of insulin-signaling; (b) screening for animals that exhibit enhanced DAF-16 activity or cytoprotective activity in the absence of insulin-signaling; and (c) identifying the agent that enhances the DAF-16 activity or cytoprotective activity. As is

discussed herein, insulin-signaling diminishes when there is insufficient food in the environment. In wild type *C. elegans*, low insulin signaling prevents AGE-1 and DAF-2 from activating AKT-1/2 to phosphorylate and inactivate DAF-16. Thus, gain-of-function mutations in *age-1*, *daf-2* and/or *akt-1/2* will result in consistently inactivated DAF-16, manifested by an inability to form dauers. In this gain-of-function embodiment, compounds are screened for their ability to promote the formation of dauer animals under starvation conditions.

In another embodiment, a method is provided for identifying an agent that increases the activity of a DAF-16 human homolog, whereby cytoprotective activity in host or human cells may be enhanced, comprising (a) contacting candidate molecules with a *C. elegans* strain comprising a *daf-16* genes that is mutated or deleted whereby functional *C. elegans* DAF-16 is not produced, a gain-of-function *daf-2*, *age-1* or *akt-1/2* mutated gene, whereby DAF-16 is inactivated even in the absence of insulin-signaling, and a *daf-16* human homolog gene; (b) screening for animals that exhibit enhanced DAF-16 homolog activity or cytoprotective activity under conditions of non-insulin signaling; and (c) identifying the agent that enhanced the DAF-16 homolog activity or cytoprotective activity. Preferably, the human *daf-16* homolog gene is provided on an extrachromosomal array. The screen would be for agents that induce the dauer state under starvation conditions.

In a preferred embodiment, the assay methods described herein additionally ensure that there is no significant perturbation or impairment in the insulin-signaling pathway (as defined herein). The purpose of the subject assays is to identify agents that act specifically on DAF-16 or human homologs thereof because these agents are very far downstream in the insulin-signaling cascade. Thus, agents that activate DAF-16 or its homologs can confer beneficial effects described herein without the detrimental effects of a general reduction or inhibition of insulin signaling. In humans, such perturbation or inhibition of the insulin-signaling pathway can cause diabetes.

The subject invention also provides a method for identifying an agent that increases the localization of DAF-16 to the nucleus in *C. elegans* cells, whereby cytoprotective activity in the cells may be enhanced, comprising (a) contacting candidate molecules with a *C. elegans* strain comprising a DAF-16::marker fusion protein encoding

construct; (b) screening for animals that exhibit localization of DAF-16::marker fusion protein in the nucleus; and (c) identifying the agent that caused localization of the DAF-16::marker protein to the nucleus. The marker can be any marker known to persons skilled in the art, including green fluorescent protein (GFP).

The subject invention also provides a method for identifying an agent that increases the expression of DAF-16 in host cells, comprising (a) contacting candidate molecules with host cells comprising a construct having a reporter gene under control of a regulatory region of *daf-16* or of a human homolog of *daf-16*; (b) screening for host cells that exhibit increased expression of the reporter; and (c) identifying the agent that caused the increased expression of the reporter. The reporter gene can be any suitable reporter gene known to skilled artisans including those encoding GFP, β-galactosidase and luciferase. The host cells may be *C. elegans* cells or cultured mammalian cells that are insulin-responsive. One method for getting the reporter gene constructs into nematodes is by injecting the construct into the germ line of adults, along with a selectable marker (such as the pRF4 plasmid which contains a dominant *rol* mutation), and then harvesting progeny that express the marker. This method makes it possible to screen for candidate molecules that cause an increase in the expression of the reporter gene and which cause an increase in the cytoprotective activities of DAF-16. Alternatively, when the host cell is human, the reporter construct would comprise the reporter gene operatively linked to a regulatory region binding site for human *daf-16* homolog. Candidate molecules would be screened to identify agents that increase the expression of the reporter gene without causing defects in the cell that result from perturbation of the insulin signaling pathway.

To identify agents that do not inhibit or perturb the insulin-signaling pathway in a human cell, the skilled artisan could look for mobilization of GLUT4 to the cell surface and increased glucose uptake. The mechanism for this activation occurs upstream of DAF-16 activation. Other assays include those for activation of Insulin Receptor (IR), PI3K and AKT. Suitable assays for activation of these upstream enzymes are known in the art.

In another embodiment, the subject invention provides an *in vitro* method of identifying an agent that inhibits AKT-1/2 interaction with DAF-16, comprising (a) contacting candidate molecules with AKT-1/2 or DAF-16; (b) adding to the mixture of

step (a), DAF-16 or AKT-1/2 respectively; and (c) identifying those agents that inhibit the interaction of AKT-1/2 and DAF-16. In such an assay, the AKT and DAF-16 or homolog proteins may be purified or recombinant. Inhibition of the interaction between the AKT proteins and the DAF-16 or homolog proteins can be detected by using standard techniques for studying the interaction of proteins, including immunoprecipitation.

The subject invention also provides an *in vitro* method of identifying an agent that inhibits the phosphorylation of DAF-16 by AKT-1/2, comprising (a) contacting candidate molecules with AKT-1/2 or DAF-16; (b) adding to the mixture of step (a), DAF-16 or AKT-2, respectively; and (c) identifying those agents that inhibit the phosphorylation of DAF-16 by AKT-1/2. The phosphorylation of DAF-16 or its homologs can be studied using standard techniques, such as the antibodies to phosphoepitopes.

In another embodiment, an *in vitro* method is provided for identifying an agent that promotes the ability of DAF-16 or DAF-16 human homologs to bind to a binding site of a regulatory region of a gene encoding a target protein, comprising (a) contacting candidate molecules with a mixture of DAF-16 or a DAF-16 human homolog and a construct comprising a reporter gene operatively linked to the DAF-16 or human homolog gene regulatory region binding site under conditions that permit *in vitro* expression; (b) detecting reporter gene expression; and (c) identifying those agents that promote DAF-16 or DAF-16 human homolog binding to the mRNA binding site. Reporter gene expression can be detected by assaying for mRNA or reporter protein produced.

In all of the above embodiments, pools of candidate molecules can be assayed for the desired activity. Each pool contains many different types of compounds. When a pool with the desired activity is identified, that pool may be fractionated or subdivided into smaller pools, and the new pools may be assayed in the same way as the parent. This cycle can be repeated until the compound with the desired activity is identified. Note, that it is possible that the desired activity may be associated with more than one candidate molecule. Thus, some of the agents of the present invention may comprise two or more compounds that function coordinately to increase DAF-16 activity.

It will be appreciated that the individual assays described above need not be mutually exclusive. Compounds initially identified in one assay (*e.g.*, increased expression of a reporter gene in mammalian cells) may be further tested in one or more of

the other assays (*e.g.*, increased cytoprotection in *C. elegans*). In this way, compounds with the most desirable activities -- such as cytoprotective activity -- and the fewest undesirable activities -- such as perturbation of the insulin signaling pathway -- can be identified. Moreover, once a compound with desirable activity has been identified, directed modifications of that compound can be made in an attempt to further increase its activity.

Compounds identified by these screens can function in a number of ways. For example, compounds that downregulate the activity of the gene products upstream from DAF-16 will cause the coordinate activation of DAF-16. Other compounds may upregulate the expression of the *daf-16* gene, leading to increased amounts of DAF-16 protein. Still other compounds may modify endogenous DAF-16 in such a way that it becomes refractory to the inhibitory activity of other gene products. For example, the compound may modify DAF-16 in such a way that it is no longer a substrate for the AKT proteins (*akt-1* and *akt-2* gene products). The AKT proteins are partially-redundant serine-threonine kinases that phosphorylate DAF-16, leading to the inactivation of DAF-16 through its translocation to the cytoplasm. Other compounds may inhibit the 14-3-3 DAF-16 interaction. Finally, it is possible that some compounds may directly activate the genes that DAF-16 controls, such as HSP70, superoxide dismutase and catalase. In preferred embodiments, the compounds of interest are those that increase DAF-16 activity -- either by increasing expression of the DAF-16 gene or increasing activity of existing DAF-16 -- without perturbing other aspects of the insulin signaling pathway. Thus, preferred compounds are those that increase the stress resistance of nematodes without giving rise to the dauer-constitutive phenotypes associated with loss-of-function mutations in the genes upstream of *daf-16*. These compounds can also be identified through the use of Northern and Western analysis of the *daf-16* gene. For example, compounds that increase the expression of *daf-16* can be identified by showing an increase in *daf-16* mRNA on a Northern blot or DAF-16 protein on a Western blot, without development of a dauer state. Where the assay is carried out in insulin-responsive human cells, activation of DAF-16 human homologs without general inhibition of the insulin-signaling pathway can be confirmed by detecting increases in transcription of *daf-16* homolog genes or reporter constructs that contain the *daf-16* Insulin Response Element (IRE), without inhibition of

activation of IR, PI3K or AKT, inhibition of glucose uptake, or inhibition of translocation of GLUT4 to the cell surface (as is discussed above).

Therapeutic Uses of Agents to Increase DAF-16 or Human Homolog Activity

Compounds identified by the methods of the instant invention have a variety of uses as therapeutic agents. In general, the compounds exert their beneficial effects by increasing the activity of DAF-16 homologs in cells. In some embodiments, this in turn leads to an increase in the transcription of genes involved in the response to stress. Thus, the compounds are useful in any disease state where tissue damage results or in aging itself. In other embodiments, the compounds of the instant invention are used to inhibit tumor growth in tumors that are comprised of the subset of cell types that respond to high levels of DAF-16 homolog-activity by inducing apoptosis. In still further embodiments, the therapeutic agent of the present invention could be a therapeutic agent preventing damage prior to the onset of a disease.

In some embodiments, the compounds of the invention are used to protect the cells of an organism from damage associated with diseases including, but not limited to, ischemia and reperfusion damages, cardiac hypertrophy, fever, inflammation, metabolic diseases, viral and bacterial infection, cell and tissue trauma, and cancer.

In other embodiments, the compounds of the invention are used to protect the cells of an organism from environmental stress, including, but not limited to, heat, cytotoxic drugs, toxins, heavy metals, asenite, anoxia, reperfusion or other damage from reactive oxidants and ethanol toxicity.

In one particularly preferred embodiment, the compounds of the instant invention are used to prevent heat-stroke in aged individuals. It is known that the rate of heat stroke is more than 10-fold higher for persons of 65 years or older as compared to younger individuals (Kilbourne at al. (1982) J. Am. Med. Assoc. 247:3332-3336). This result is paralleled by studies indicating that cells isolated from aged organisms are much more sensitive to heat shock than comparable cells isolated from younger organisms. The increased sensitivity of aged cells is in part due to the decrease in the inducibility of heat shock proteins, including HSP72, in response to thermal stress (Volloch et al. (1998) Cell Stress and Chaperones 3:265-271). Forced expression of HSP72 in aged cells can restore

thermotolerance. These results indicate that the compounds of the instant invention, which induce heat-shock gene expression, can be used to increase thermotolerance in aged individuals.

In other embodiments, the compounds of the instant invention are used to treat viral infections. It is known that certain compounds, such as cyclopentenone prostanoids, can endow cells with potent antiviral activity against a number of DNA and RNA viruses, including HIV-1 (Rozera, C. et al. (1996) J. Clin. Invest. 97:1795-1803). The effects of such compounds are mediated through the activation of heat-shock gene transcription, including the HSP70 gene. The heat shock proteins are involved at multiple levels in the viral replication pathway. Because increased DAF-16 homolog activity leads to an increase in HSP70 levels, the compounds of the present invention are useful as novel antiviral agents. Thus, the compounds of the invention can act in two different ways to protect cells from viral infection: 1) inhibiting viral replication; and 2) protecting cells from the damage caused by viral activity. In other embodiments of the invention, such compounds would prevent damage resulting from stress resulting from surgery and/or from anesthetic action or removal or recovery from surgery or anesthesia.

In other embodiments, the compounds of the instant invention are used to suppress the growth and development of tumors. As described above, in certain cell types, active DAF-16 homologs -- such as FRHKL1 -- activate the transcription of apoptosis-promoting genes, such as the Fas ligand. Tumors formed from cell types that respond to increased DAF-16 homolog activity by inducing apoptosis can be treated with the agents of the instant invention. In additional embodiments, the compounds of this invention prevent necrosis. In still further embodiments, the compounds of this invention prevent damage associated with lack of function due to inactivity, coma, etc.

Compounds isolated according to the methods of the invention can be administered to patients for the therapeutic purposes outlined above in a pharmaceutically acceptable excipient, diluent, or carrier. Any method for administration is contemplated, including, but not limited to, oral, intravenous, subcutaneous, and intramuscular administration. The compounds can be administered to either treat an existing disease or they can be administered prophylactically.

Increasing the Dosage of DAF-16 Homologs in Human Cells

In one embodiment of the invention, the dosage of one or more DAF-16 homologs is increased in human cells. In preferred embodiments, this can be done by isolating DNA that encodes the DAF-16 homolog(s), and splicing the isolated DNA into a vector using any of the techniques well known in the art. Suitable protocols are disclosed, for example, in Ausubel et al., Current Protocols in Molecular Biology, 1996, Wiley & Sons, New York, NY. In order to obtain expression of the DAF-16 homolog, the vector also contains a promoter sequence located in such a way that the isolated DNA falls under its transcriptional control. In some embodiments, the promoter is a general activator of transcription; hence, the vector can direct the expression of the DAF-16 homolog DNA in a large number of different cell types. In other embodiments, the promoter directs the expression of the DAF-16 homolog in a particular subset of tissues, a particular tissue, a small number of cell types, or even in a single cell type. A very wide variety of promoters are known in the art, and the skilled artisan can select and manipulate promoters in order to obtain the desired expression pattern for the operatively-associated DAF-16 homolog DNA. In addition, it may be advantageous to express the DAF-16 homolog as a fusion with another protein.

In preferred embodiments, the DAF-16 homologs are expressed at a sufficiently high level that the AKT kinases are not able to phosphorylate -- and hence inactivate -- all of the DAF-16 homolog. In other embodiments, the constructs express mutated versions of DAF-16 homologs that are unable to respond to AKT phosphorylation. Such mutated DAF-16 homologs would be constitutively active.

In order to obtain expression of the DAF-16 homolog constructs provided above, it will be appreciated by those skilled in the art that it is necessary to introduce the vector into the human body in such a way that the vector DNA can be assimilated by cells. Numerous methods are known in the art of gene therapy. In one embodiment, the DAF-16 homolog constructs are encapsulated within a virus, and the virus is introduced into the human body. Once within the body, the virus can infect cells, and deliver its DAF-16 construct virion to those cells. The virus can be chosen so that it attaches only to specific cells, and so the DAF-16 homolog construct can effectively be targeted to those particular cells.

The above methods can be used in any medical application where it would be desirable to increase the level of stress response proteins in a tissue or cell type. By way of example only, one application involves expressing the DAF-16 homolog construct in myocardial cells to protect them from ischemic stress and damage by reperfusion. For example, a patient who requires heart surgery can have the dosage of DAF-16 homologs in the heart increased by the above-mentioned method prior to surgery. Because the heart muscle will have increased expression of stress response proteins, damage sustained by the heart during surgery can be minimized.

In other embodiments, the constructs of the instant invention are used to suppress the growth and development of tumors. In certain cell types, the DAF-16 homolog FKHRL1, when unphosphorylated, and hence active as a transcription factor, can activate the transcription of genes involved in cell death, including the Fas ligand. This activity of FKHRL1 is inhibited by the kinase AKT, which phophorylates FKHRL1, and leads to its translocation to the cytoplasm. It has been suggested that the induction of cell death is important in the suppression of tumor development, leading to the death and engulfment of nascent tumor cells in a process known as apoptosis. The constructs of the instant invention, although conferring cytoprotective abilities on some cell types, may induce cell death in other types of cell. This difference in response to DAF-16 homologs may result either from intrinsic differences in the way different cells respond to DAF-16 homolog activity. Alternatively, it may be a dose-dependent effect wherein the levels of DAF-16 activity required for promoting apoptosis and cytoprotection are markedly different. In any case, tumors formed from cell types that respond to increased DAF-16 homolog activity by inducing apoptosis can be treated with the constructs of the instant invention. For example, a construct for the over-expression of a DAF-16 homolog can be encapsulated within a virus that binds specifically to the cell types that comprise the tumor. In this way, the over-expression construct is delivered to the tumor. In the present invention, the beneficial effects obtained from the up-regulation or activation of DAF-16 or the production or administration of DAF-16 homologs is useful for the treatment of all diseases or medical conditions where oxidative stress is implicated as a causative or extenuating factor.

EXAMPLES

EXAMPLE 1 - Methods and Materials

The following methods and materials were used in subsequent Examples.

A. Strain construction.

Strains were purchased from either the *C. elegans* Genetic Center (University of Minnesota, Minneapolis, MN) or from the Johnson lab (University of Colorado, Boulder, CO). Nematodes were cultured under standard conditions (Brenner, S. (1974) Genetics 77:71-94). Strains included: N2 (wild type), TJ1052 (*age-1(hx546)*), and GR1307 (*daf-16(mgDf50)*, a strain lacking all *daf-16* coding regions). Transgenic nematodes were derived by microinjection of test DNA at 20 µg/ml and pRF4 (transformation marker) at 100 µg/ml (Mello, C. et al. (1991) Embo J. 10:3959-70). Cosmid R13H8 was obtained from The Sanger Center (UK). Strains are named as parent strain then extrachromosomal array (Ex). All transgenics carried pRF4. For experiments involving sterile animals, sterility was defined as adult animals that lacked oocytes and eggs. Life span was determined on seeded NGM (nematode growth media) plates at either 20°C or 25°C, as indicated. Animals were considered dead if no pharyngeal pumping was evident and they failed to respond to repeated prodding (Johnson T. et al. (1982) PNAS 79:6603-7).

B. Double stranded RNAi

RNA was isolated from mixed stage, wild type (N2) *C. elegans* using Trizol (Sigma). RNA was used as template for random primed cDNA synthesis using M-MuLV Reverse Transcriptase (New England Biolabs). The single strand cDNA was amplified with *daf-16* specific primers; STH14 (atcccgggctagtgatgatggtggtgatggtgcaaatcaaaatgaatatgctgccc) and STH17 (tggatccccatgatggagatgctggtagat). The PCR product was cloned into a PCR cloning vector pSTBlue (Novagen). DNA fragments derived from this construct was used in two in vitro transcription reactions, using either T7 or SP6 RNA polymerase (Ambion). Separate single stranded RNAs were mixed and injected into the gonad. F1 progeny were examined for phenotypes.

C. Thermotolerance, UV tolerance, oxidative stress tolerance and life span

Thermotolerance Intrinsic thermotolerance was measured as percent of a cohort of staged 3 day old adult worms that survive a near-lethal heat shock. Specifically, 30-40

transgenic adults were placed on seeded NGM plate, and left to lay eggs for 3-4hrs. Adults were removed and eggs were allowed to develop until 3 days past L4 molt. 30-40 rolling adults were placed on a small seeded NGM plate, and the plate was wrapped in parafilm and floated in a circulating water bath set at 37.5°C for 2 hours. Parafilm was then removed and worms were allowed to recover at 20°C for 24hrs, then scored for viability. Life span was determined as described (Johnson, T. et al. (1982) PNAS 79:6603-7). Due to variables, such as plate thickness and rearing temperature, thermotolerance comparisons were only made for strains done in parallel.

UV tolerance. 30-40 staged three day old adult animals were removed from a seeded plate, washed in 1XS-Basal, then transferred to an unseeded NGM plate. Animals were exposed to 400J/m2 in a Stratalinker 2400 (Stratagene). Animals were removed from the unseeded plate and placed on a seeded one. Life span was calculated from the day of UV treatment. UV treatment often led to egg laying defects and bagged adults. These animals were censored from life span calculations.

Daily heat shock. Plates containing 30-40 animals were wrapped in parafilm and floated in a circulating water bath at 34°C for 1hr, separated by 24 hour periods. Parafilm was removed and animals returned to 20°C. Animals were scored for viability immediately before the heat shock.

Oxidative stress. Since oxidative stress frequently results in a large number of bagged animals, only purged animals were examined for resistance to oxidative stress. Thirty to forty staged 5-day-old adult animals were exposed to 0.3% hydrogen peroxide for 40 minutes. Survival was calculated from the day of peroxide treatment. Life spans were determined in parallel for each strain. Data in Figure 2 are from one representative experiment and have been replicated. Animals were considered sterile if no oocytes or eggs were present as adults.

D. DAF-16::GFP Plasmid Construction.

The DAF-16::GFP plasmid was constructed as follows: primers STH6 (ccgcggccgcgaattcaacttgagcatctcttttttcttgg) and STH24 (ccactagtgagttctctggaattgg) were used to amplify a six kilobase fragment upstream of the *daf-16* gene using cosmid R13H8 as a template. This fragment was digested with *Not*I and *Spe*I and cloned into *Not*I/*Spe*I

digested pBluescript II SK(-) (Stratagene, Inc.) to create pGP10. pGP10 was digested with *Not*I/*Spe*I, the ends blunted with T4 polymerase and cloned into pPD95.81, which had been digested with *Bam*HI and ends blunted with T4 polymerase, to create pGP12. Primers STH17 (tggatccccatgatggagatgctggtagat) and STH32 (ctaccggtggcaaatcaaaatgaatatgctgccc) were used to amplify *daf-16a* coding region from single stranded cDNA derived from mixed stage N2 RNA. STH12/STH32 PCR product was digested with *Bam*HI and *Age*I then ligated into *Bam*HI/*Age*I digested pGP12 to create pGP30 (DAF-16::GFP).

E. FKHR::luciferase Plasmid Construction.

Oligonucleotides corresponding to a KPNI to XhoI fragment (ggtaccgagctctaagcaaaacaagccagcaaaacaagtccgcaaaacaagtactcgag), with three consensus insulin response sequences (IRS) (caaaacaa) were hybridized and cut with KpnI and XhoI and cloned into KpnI/XhoI digested pGL3 Promoter (Promega). This construct has a minimal SV40 promoter and the luciferase coding region (Luc+). Binding of DAF-16 family member will activate transcription of the luciferase reporter.

F. Western Analysis for HSP70, HSP90 and Ubiquitin.

For each of the panels in Figure 6, five animals of the appropriate genotype were placed in 5 µl of sterile water and rapidly frozen in a -70°C isopropanol bath. For lysis, 2 µl of LDS loading buffer and 1 µl of reducing agent (10 mM dithiothreitol) were added to the tube, which was then boiled for 10 minutes. Samples were loaded onto a 4-12% Bis-Tris gel (Novex, Inc.) and then blotted to nitrocellulose. Anti-HSP70, anti-HSP-90 and anti-ubiquitin (Sigma, Inc.) were used for probing and detection was done with ECL (Enhanced Chemi-Luminescence, Amersham, Inc.). The HSP70 Western analysis used antibodies to *Drosophila* HSP70 (Sigma, Inc.), which recognizes both constitutive and inducible forms of HSP70 from many species, but does not detect any *E. coli* proteins. Densitometry measurements were done using Scion Image (Scion, Inc.).

EXAMPLE 2 - Multiple copies of R13H8 confer stress resistance on transgenic nematodes.

Given the central role of *daf-16* in long-lived nematode mutants, the effect of multiple copies of *daf-16* on stress resistance and life span was tested. The *daf-16* gene is contained wholly within the cosmid R13H8, and this cosmid will rescue *daf-16(lf)* mutations (Ogg, S. et al. (1997) Nature 389:994-9; and Lin, K. et al. (1997) Science 278:1319-22). A mix of R13H8 and pRF4 were injected to establish several independent transgenic lines (EX13H8) in both N2 (wild type) and the long lived strain TJ1052 (*age-1(hx546)*). Independent lines from each series of injections throw a varying percentage of sterile animals (Table 2). These animals show a range of germ line phenotypes from no germ cells to weakly proliferating germ cells. This phenotype is similar to *daf-2(ts)* alleles when shifted to the non-permissive temperature at L3-L4 (Gems D. et al. (1998) Genetics 150:129-55), yet a role for *daf-16* in the germ line had not been previously noted. Furthermore, at lower temperatures a higher frequency of sterile animals is seen (see Table 2).

As a test for stress resistance, an assay based on the survival fraction of transgenic nematodes lines exposed to a near-lethal heat shock was used (see Materials and Methods). The intrinsic thermotolerance for different strains was assessed by varying the exposure time for different strains. For example, wild-type (N2) and animals transgenic for the transformation marker alone (N2;ExpRF4) have low survival rates when exposed to 37.5°C for 2 hours, while strains known for increased longevity and stress resistance do well (Figure 2a). Both sterile and fertile animals from several independent lines of animals carrying an extrachromosomal array of cosmid R13H8 (N2; Ex R13H8) were tested. Sterile animals derived from multiple independent lines of N2;Ex R13H8 exhibit a strong increase in thermotolerance (see Figure 2a).

Next, survival was tested relative to *age-1(hx546)* by increasing the time of exposure to lethal temperatures. A 37.5°C heat shock for 2.5hr results in low survivability even for known thermotolerant strains. Only about 5% of *age-1(hx546)* survived this heat shock. The sterile class of animals with increased dosage of cosmid R13H8 had much higher survivals of 80% or greater under the same conditions (see Figure 2b).

Long-lived strains of *C. elegans* are more resistant to ultraviolet light (UV) (Murakami, S. et al. (1996) Genetics 143:1207-18). Sterile animals derived from N2;EXR13H8 and *age-1(hx546)*;ExR13H8 were tested for UV resistance using the method described in Materials and Methods. ExR13H8 increased mean survival after UV treatment of both wild type (increase about 33% versus N2; Figure 2c) and *age-1(hx546)* (increase about 37% over *age-1 (hx546)*;ExpRF4 strains; Figure 2d).

Resistance to oxidative damage, one of the leading causes of accumulated cellular damage (for reviews, see Stadtman, E. (1992) Science 257:1220-4; and Beckman, K. et al. (1998) Physiol. Rev. 78:547-81), could be altered as well. Sterile animals derived from ExR13H8 strains were tested (see Materials and Methods) to determine whether they were resistant to hydrogen peroxide. Consistent with thermal and ultraviolet light resistance, sterile ExR13H8 animals showed remarkable resistance to peroxide treatment (Figure 2e).

EXAMPLE 3 - Increased Thermotolerance is due to *daf-16.*

To determine if the strong increase in thermotolerance is due to *daf-16,* the effect of injection of dsRNA specific to *daf-16* coding regions on thermotolerance and frequency of steriles was tested (see Materials and Methods). Injection of dsRNA specifically inhibits the activity of the gene injected, a process called RNAi (for overview see Tabara et al. (1998) Science 282:430-1). The occurrence of steriles and increase in thermotolerance in progeny of injected animals was tested. The *age-1(hx-546)*;ExR13H8 strains were injected with dsRNA because of the high frequency of steriles at 20°C. Injection of dsRNA reduces the occurrence of steriles, but will not completely eliminate them (see Table 3). Even though sterile animals were produced, they no longer showed any increase in thermotolerance (see Table 3). Since RNAi is thought to mimic loss of function of the target gene, this result suggests that the increased thermotolerance is due to increased levels of *daf-16* transcript or protein, and not another gene on the cosmid.

EXAMPLE 4 - Increased dosage of *daf-16* and life span.

It was next determined whether an increased copy number of cosmid R13H8 would increase mean life span of transgenic nematodes relative to controls. First, unselected transgenics, with no bias towards fertility or sterility, were tested. Under constant

temperature, no increase in life span was observed between N2;ExpRF4 and unselected N2;ExR13H8 strains (see Table 4). Given the strong stress-resistant phenotype of sterile N2;ExR13H8 animals, the life spans of sterile animals and fertile animals derived from N2;ExR13H8, versus controls were examined (see Table 4). No significant extension of mean life span was observed.

A mild heat shock will increase the life span of *C. elegans* as well as several other species (Shama, S. et al. (1998) Exp. Cell Res. 245:379-88; and Rattan, S. (1998) Biochem. Mol. Biol. Int. 45:753-9). An increase in life span of ExR13H8 animals had been noted by the inventors when the animals were maintained in a poorly controlled temperature environment (data not shown). Therefore, the effect of mild daily heat shock on life span of wild type *C. elegans* and those carrying extra copies of R13H8 was examined. A 1 hour 34°C heat treatment every twenty four hours (see Materials and Methods) increased mean life span of wild type *C. elegans* approximately 15% (see Figure 3), but did not increase maximum life span, due to increased late life lethality. Surprising, a daily heat shock dramatically increased mean and maximum life span of animals carrying extrachromosomal arrays of cosmid R13H8 (see Figure 3). Both mean and maximum life span were increased (27% and 32% respectively) versus N2;ExpRF4 heat shocked animals.

EXAMPLE 5 - DAF-16::GFP fusion protein becomes nuclear localized upon heat shock.

Since ExR13H8 animals showed an increase in life span when given a mild heat shock, the sub-cellular distribution of DAF-16 after such treatment was examined. In mammalian systems, insulin-like signaling represses the activity of DAF-16 homologues by inhibiting entry into the nucleus (Brunet, A. et al. (1999) Cell 96:857-68; Durham, S. et al. (1999) Endocrinology 140:3140-6; Biggs, W. et al. (1999) PNAS 96:7421-6; Guo, S. et al. J. Biol. Chem. 274:17184-92; Rena, G. et al. (1999) J. Biol. Chem.274:17179-83; Tang, E. et al. (1999) J. Biol. Chem. 274:16741-6; and Nakae, J. et al. J. Biol. Chem. 274:15982-5). To conduct localization studies in the nematode, a DAF-16::GFP reporter construct was made which fuses GFP to the predicted last amino acid (510) of the DAF-16a protein, and which is driven by six kilobases of sequence upstream of *daf-16* (see Materials and Methods).

Consistent with previous studies (Ogg, S. et al. (1997) Nature 389:994-9), it was found that under normal culture conditions, the fusion protein is largely cytoplasmic (Figure 4b). A mild heat shock of one hour at 34°C resulted in dramatic nuclear localization (Figure 4d).

EXAMPLE 6 - DAF-16::GFP fusion protein increases life span and stress resistance.

Animals transgenic for DAF-16::GFP arrays (ExDAF-16::GFP) were tested to determine if they were longer-lived and more stress resistant. Because ExDAF-16:GFP animals do not segregate animals with weak germ line proliferation (as do ExR13H8 animals), unselected ExDAF-16::GFP animal could be tested. It was found that ExDAF-16:GFP animals were more thermotolerant (see Materials and Methods and Figure 5a) and UV resistant (see Materials and Methods, and Figure 5b) than ExpRF4 animals. Additionally, it was found that DAF-16::GFP resulted in slight increases in life span relative to ExpRF4 strains at 20°C (Figure 5c).

EXAMPLE 7 - Construct useful in identifying agents that up-regulate human homologs of DAF-16

A construct that can be useful in identification of agents that up-regulate human homologs of DAF-16, such as FKHR, is illustrated in Figure 10. The construct contains three insulin response sequences (IRS) upstream of a minimal promoter and the luciferase gene (see Materials and Methods). The IRS is a binding site for DAF-16 or its human homologs (Furayama, F. et al. (2000) Biochem. J. 349:629-634). The construct can be transfected into human cell lines and stable transformants selected. The construct is likely to have some low level of background activity. In a cell based assay system, candidate agents can be screened for those that increase transcription of the luciferase. These compounds can be useful in up-regulating activity of FKHR or other DAF-16 human homologs.

EXAMPLE 8 - Increased dosage of *daf-16* leads to high levels of HSP70 and Ubiquitin

Generally, as an organism ages, transcripts of several stress inducible genes increase in abundance, which has been interpreted to be a response to accumulated cellular

damage (Lee, C. et al. (1999) Science 285:1390-3; and Ly, D. et al. (2000) Science 287:2486-92). HSP70 proteins are abundant, highly conserved stress proteins induced by the presence of mis-folded proteins. In the present example, it was found that HSP70 protein levels increase in wild-type nematodes as they age (Figure 6). In Figure 6a, each lane contains extracts from five adults of the indicated age. The optical density (Scion Image, Scion, Inc.) of each lane is shown in Figure 6b. It was found that the life span of wild type was slightly greater than that of the *daf-16(mgDf50)* strain, which lacks all *daf-16* coding regions (Figure 6c). Deletion of *daf-16* resulted in an earlier induction of HSP70 (Figure 6a) suggesting that in the absence of *daf-16*, damage accumulates more rapidly. Since *C. elegans* normally lives only a few days in the wild, it is unlikely that large amounts of resources are devoted to recycling and repairing of cellular components. This could explain why removal of *daf-16* has little effect on life span.

To identify possible mechanisms for the increased thermotolerance phenotype of ExR13H8 strains, the levels of HSP70 and HSP90 proteins were examined in wild type and transgenic strains. The levels of HSP90 were unchanged in all of the strains (Figure 7a). However, sterile animals carrying arrays of R13H8 had much higher levels of HSP70 than did animals with transformation marker alone (see Figure 7b). The levels of HSP70 were slightly higher in *age-1(hx546)* animals versus wild type, but the levels in N2;ExR13H8 strains were approximately three-fold higher than in ExpRF4 strains.

In yeast, the ubiquitin gene is required for thermotolerance (Finley, D. et al. (1987) Cell 48:1035-46). Levels of ubiquitin and ubiquitinated proteins in strains carrying increased dosage of *daf-16* were examined using an antibody to the highly conserved ubiquitin protein, which recognized ubiquitin as well as ubiquitinated proteins. In sterile animals derived from both N2;ExR13H8 and *age-1(hx-546)*;ExR13H8 strains, increased levels of ubiquitin, and associated ubiquitinated proteins were observed (Figure 7c). Consistent with the increased stress resistance of ExDAF-16::GFP strains, it was also found that HSP70 and ubiquitin levels were elevated in these animals as well (Figure 7d and 7e).

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made to the invention without departing from the spirit and scope of the invention.

Tables

Table 1. Life span and stress resistance of wild type and *daf-16(mgDf50)*.

Genotype	Life expectancy (replicates)#	UV resistance (replicates)#	Thermotolerance*
wild-type	15.3 ±0.8 (2)	5.3 ± 0.27 (2)	0.20 ± 0.09(2)
daf-16(mgDf50)	14.0 ± 0.6 (2)	5.6 ± 0.26 (2)	0.15 ± 0.08(2)

#Data shown is from one representative experiment at 25ºC, ± SEM. UV dose 400J/m2.
*Fraction surviving a 37.5ºC heat shock for 1 hr 45 min.

Table 2. Sterile adults in transgenic lines.

Strain	15ºC	20ºC	25ºC
N2; ExpRF4	2	0	0
N2; ExR13H8(1)	63	23	2
N2; ExR13H8(2)	16	14	3

Number of sterile adults out of 100 adults tested for each strain and temperature.

Table 3. *daf-16* RNAi

Strain	*daf-16* RNAi	Survival ± SEM*	n	% Steriles	n
age-1(hx546); ExR13H8	No	0.97 ±0.03	33	46	195
age-1(hx546); ExR13H8	Yes	0.09 ± 0.06	23	28	94

*Survival of a 37.5ºC heat shock for 2.5 hours. RNA was cloned and injected into nematodes as described in Materials and Methods.

Table 4. ExR13H8 life spans

Strain	Mean life span (replicates)#
N2; ExpRF4	16.74+0.4(2)
N2; ExR13H8(1)	16.70+0.3(2)
N2; ExR13H8(2)	16.56+0.4(2)
N2; ExpRF4	15.64+0.5(2)
N2; ExR13H8(Fer)	16.31+0.5(2)
N2; ExR13H8(Str)	17.77+0.5(2)

#Data shown is from one representative experiment at 25ºC, ± SEM.

Claims:

1. A method of identifying an agent that enhances the activity of DAF-16 or a DAF-16 human homolog, comprising:

a) contacting DAF-16 or a DAF-16 human homolog with candidate agents under assay conditions for DAF-16 activity or DAF-16 human homolog activity; and

b) identifying those agents that enhance the activity of the DAF-16 or DAF-16 human homolog.

2. The method of claim 1, wherein the identified agent does not inhibit an insulin-signaling pathway.

3. The method of claim 1, wherein the activity of DAF-16 or a DAF-16 homolog comprises activation of a molecule selected from the group consisting of HSP70, HSP90, catalase, ubiquitin and superoxide dismutase.

4. The method of claim 1, wherein the activity of DAF-16 or the DAF-16 homolog arises from improved *daf-16* or homolog gene transcription, improved *daf-16* mRNA or homolog mRNA translation, improved DAF-16 or DAF-16 homolog activation, improved target protein activation, or any combination thereof.

5. The method of claim 1, wherein the DAF-16 homolog is selected from the group consisting of AFX, FKHR, and FKHRL1.

6. A method of identifying an agent that enhances the activity of DAF-16 or a DAF-16 human homolog, comprising:

a) contacting *daf-16* or a *daf-16* human homolog with candidate agents under conditions permitting expression;

b) conducting an assay for DAF-16 activity or DAF-16 human homolog activity; and

c) identifying those agents that enhance the activity of the DAF-16 or DAF-16

human homolog.

7. The method of claim 6 wherein the identified agent does not inhibit an insulin-signaling pathway.

8. The method of claim6, wherein the activity of DAF-16 or a DAF-16 homolog comprises activation of a molecule selected from the group consisting of HSP70, HSP90, catalase, ubiquitin and superoxide dismutase.

9. The method of claim6, wherein the activity of DAF-16 or the DAF-16 homolog arises from improved *daf-16* or homolog gene transcription or translation, improved DAF-16 or DAF-16 homolog activation, improved target protein activation, or any combination thereof.

10. The method of claim6, wherein the DAF-16 homolog is selected from the group consisting of AFX, FKHR, and FKHRL1.

11. A method for identifying an agent that increases the activity of DAF-16 in *C. elegans* cells, whereby cytoprotective activity in the cells may be enhanced, comprising:

a) contacting candidate molecules with a *C. elegans* strain comprising a wild type *daf-16* locus;

b) screening for animals that exhibit enhanced DAF-16 activity or cytoprotective activity; and

c) identifying the agent that enhanced the DAF-16 activity or cytoprotective activity.

12. The method of claim 11, wherein the identified agent does not inhibit an insulin-signaling pathway.

13. The method of claim 11 wherein the cytoprotective activity is selected from the group consisting of improved UV resistance, improved thermotolerance, improved

oxidative stress resistance and improved life span.

14. The method of claim 11 wherein the activity comprises activation of a molecule selected from the group consisting of HSP70, HSP90, catalase, ubiquitin and superoxide dismutase.

15. The method of claim 11 wherein the increased activity of DAF-16 arises from improved transcription, improved translation, improved protein activation or any combination thereof.

16. A method for identifying an agent that increases the activity of a DAF-16 human homolog, whereby cytoprotective activity in host or human cells may be enhanced, comprising:

a) contacting candidate molecules with a *C. elegans* strain comprising a *daf-16* gene that is mutated or deleted whereby functional *C. elegans* DAF-16 is not produced, and a *daf-16* human homolog gene;

b) screening for animals that exhibit enhanced DAF-16 human homolog activity or cytoprotective activity; and

c) identifying the agent that enhanced the DAF-16 human homolog activity or cytoprotective activity.

17. The method of claim 16 wherein the identified agent does not inhibit an insulin-signaling pathway.

18. The method of claim 16 wherein the cytoprotective activity is selected from the group consisting of improved UV resistance, improved thermotolerance, improved oxidative stress resistance and improved life span.

19. The method of claim 16 wherein the cytoprotective activity comprises activation of a molecule selected from the group consisting of HSP70, HSP90, catalase, ubiquitin and superoxide dismutase.

20. The method of claim 16 wherein the DAF-16 human homolog is selected from the group consisting of AFX, FKHR and FKHRL1.

21. The method of claim 16 wherein the increased activity of the DAF-16 homolog arises from improved transcription, improved translation, improved protein activation or any combination thereof.

22. The method of claim 21 wherein the improved protein activation comprises reduced phosphorylation of DAF-16 by AKT-1/2.

23. A method for identifying an agent that increases the activity of DAF-16 in *C. elegans* cells, whereby cytoprotective activity in the cells may be enhanced, comprising:

a) contacting candidate molecules with a *C. elegans* strain having a gain-of-function *daf-2*, *age-1* or *akt-1/2* mutation, whereby DAF-16 is inactivated even in the absence of insulin-signaling;

b) screening for animals that exhibit enhanced DAF-16 activity or cytoprotective activity in the absence of insulin-signaling; and

c) identifying the agent that enhances the DAF-16 activity or cytoprotective activity.

24. The method of claim 23 wherein the identified agent does not inhibit an insulin-signaling pathway.

25. The method of claim 23 wherein the cytoprotective activity is selected from the group consisting of improved UV resistance, improved thermotolerance, improved oxidative stress resistance and improved life span.

26. The method of claim 23 wherein the cytoprotective activity comprises activation of a molecule selected from the group consisting of HSP70, HSP90, catalase, ubiquitin and superoxide dismutase.

27. The method of claim 26 wherein the increased activity of DAF-16 arises from improved transcription, improved translation, improved DAF-16 protein activation or improved target protein activation.

28. A method for identifying an agent that increases the activity of a DAF-16 human homolog, whereby cytoprotective activity in host or human cells may be enhanced, comprising:

a) contacting candidate molecules with a *C. elegans* strain comprising a *daf-16* genes that is mutated or deleted whereby functional *C. elegans* DAF-16 is not produced, a gain-of-function *daf-2*, *age-1* or *akt-1/2* mutated gene, whereby DAF-16 is inactivated even in the absence of insulin-signaling, and a *daf-16* human homolog gene;

b) screening for animals that exhibit enhanced DAF-16 homolog activity or cytoprotective activity under conditions of non-insulin signaling; and

c) identifying the agent that enhanced the DAF-16 homolog activity or cytoprotective activity.

29. The method of claim 28 wherein the identified agent does not inhibit an insulin-signaling pathway.

30. The method of claim 28, wherein the cytoprotective activity is selected from the group consisting of improved UV resistance, improved thermotolerance and improved life span.

31. The method of claim 28, wherein the DAF-16 homolog activity comprises activation of a molecule selected from the group consisting of HSP70, HSP90, catalase, ubiquitin and superoxide dismutase.

32. The method of claim 28, wherein the DAF-16 homolog is selected from the group consisting of AFX, FKHR and FKHRL1.

33. The method of claim 28, wherein the increased activity of the DAF-16 homolog arises from improved transcription, improved translation, improved DAF-16 homolog activity or improved DAF-16 target protein activation.

34. The method of claim 28, wherein the improved target protein activation comprises reduced phosphorylation of DAF-16 homolog phosphorylation by AKT-1/2.

35. A method for identifying an agent that increases the localization of DAF-16 to the nucleus in *C. elegans* cells, whereby cytoprotective activity in the cells may be enhanced, comprising:

a) contacting candidate molecules with a *C. elegans* strain comprising a DAF-16::marker fusion protein encoding construct;

b) screening for animals that exhibit localization of DAF-16::marker fusion protein in the nucleus; and

c) identifying the agent that caused localization of the DAF-16::marker protein to the nucleus.

36. The method of claim 35 wherein the identified agent does not inhibit an insulin-signaling pathway.

37. The method of claim 35 wherein the marker is selected from the group consisting of green fluorescent protein (GFP), β-galactosidase and luciferase.

38. A method for identifying an agent that increases the expression of DAF-16 in host cells, comprising:

a) contacting candidate molecules with host cells comprising a construct having a reporter gene under control of a regulatory region of *daf-16* or of a human homolog of *daf-16*;

b) screening for host cells that exhibit increased expression of the reporter; and

c) identifying the agent that caused the increased expression of the reporter.

39. The method of claim 38 wherein the identified agent does not inhibit an insulin-signaling pathway.

40. The method of claim 38, wherein the reporter gene encodes a protein selected from the group consisting of green fluorescent protein (GFP), β-galactosidase and luciferase.

41. The method of claim 38, wherein the host cells are *C. elegans* cells.

42. The method of claim 38, wherein the host cells are cultured mammalian cells that are insulin-responsive.

43. An *in vitro* method of identifying an agent that inhibits AKT-1/2 interaction with DAF-16, comprising:

a) contacting candidate molecules with AKT-1/2 or DAF-16;

b) adding to the mixture of step a), DAF-16 or AKT-1/2 respectively; and

c) identifying those agents that inhibit the interaction of AKT-1/2 and DAF-16.

44. The method of claim 43 wherein the identified agent does not inhibit an insulin-signaling pathway.

45. An *in vitro* method of identifying an agent that inhibits the phosphorylation of DAF-16 by AKT-1/2, comprising:

a) contacting candidate molecules with AKT-1/2 or DAF-16;

b) adding to the mixture of step a), DAF-16 or AKT-2, respectively; and

c) identifying those agents that inhibit the phosphorylation of DAF-16 by AKT-1/2.

46. The method of claim 43 wherein the identified agent does not inhibit an insulin-

signaling pathway.

47. An *in vitro* method of identifying an agent that promotes the ability of DAF-16 or DAF-16 human homologs to bind to a binding site of a regulatory region of a gene encoding a target protein, comprising:

a) contacting candidate molecules with a mixture of DAF-16 or a DAF-16 human homolog and a construct comprising a reporter gene operatively linked to the DAF-16 or human homolog gene regulatory region binding site under conditions that permit *in vitro* expression;

b) detecting reporter gene expression; and

c) identifying those agents that promote DAF-16 or DAF-16 human homolog binding to the mRNA binding site.

48. The method of claim 47 wherein the identified agent does not inhibit an insulin-signaling pathway.

49. The method of claim 47, wherein the target protein is selected from the group consisting of HSP70, HSP90, catalase, ubiquitin and superoxide dismutase.

50. The method of claim 47, wherein the reporter gene is selected from the group consisting of green fluorescent protein (GFP), β-galactosidase and luciferase.

51. The method of claim 47, wherein the DAF-16 human homolog is selected from the group consisting of AFX, FKHR and FKHRL1.

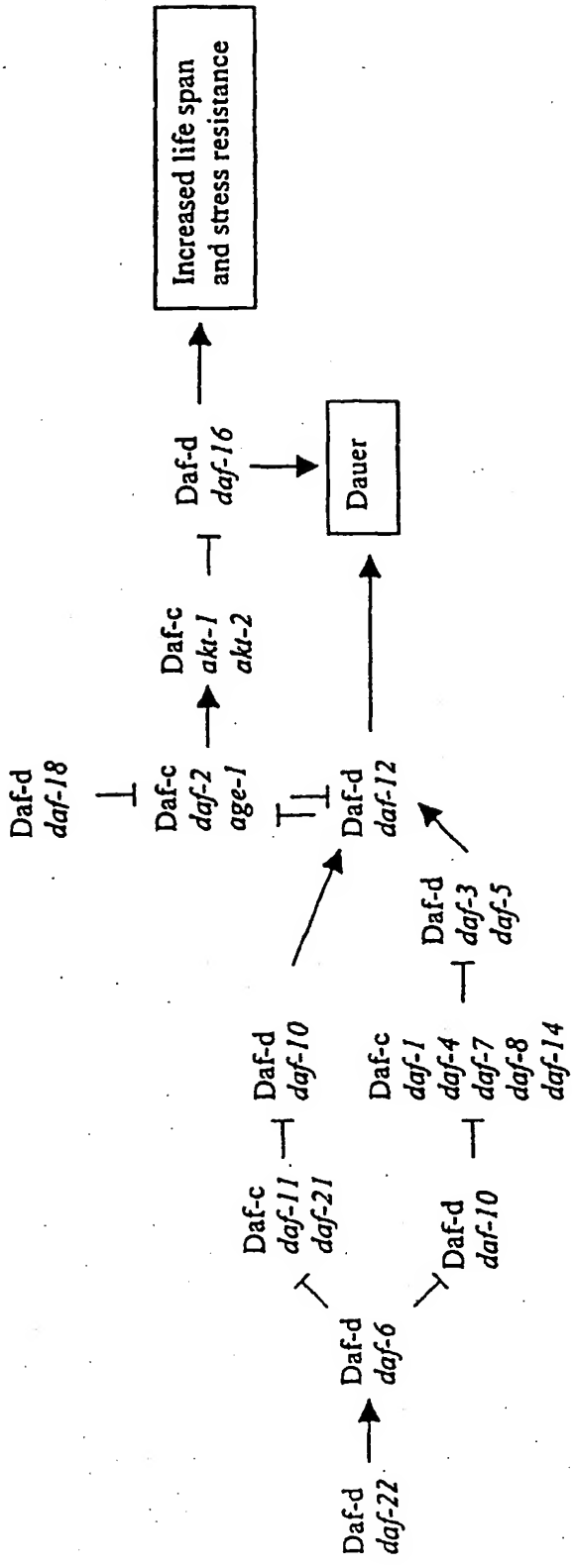
Increased life span and stress resistance
Daf-d daf-16
Dauer
Daf-c akt-1 akt-2
Daf-d daf-18
Daf-c daf-2 age-1
Daf-d daf-12
Daf-d daf-3 daf-5
Daf-d daf-10
Daf-c daf-11 daf-21
Daf-c daf-1 daf-4 daf-7 daf-8 daf-14
Daf-d daf-10
Daf-d daf-6
Daf-d daf-22

Fig. 1

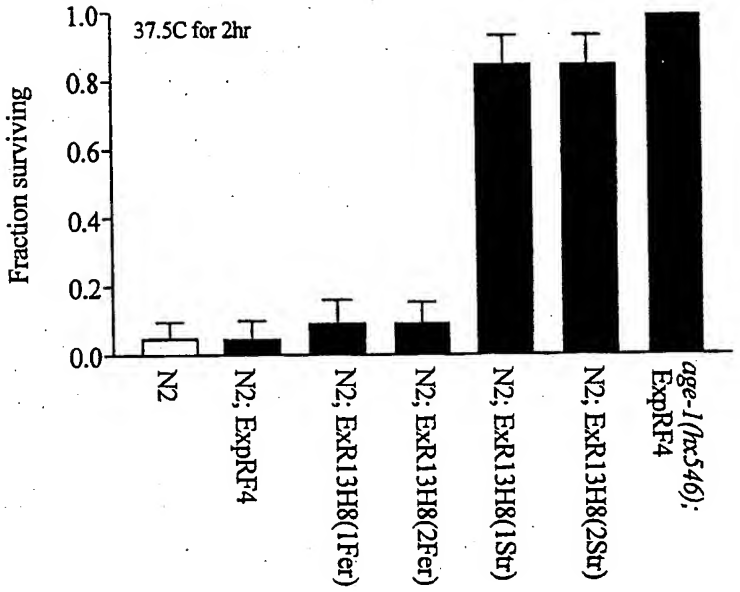
37.5C for 2hr
Fraction surviving
1.0
0.8
0.6
0.4
0.2
0.0
N2
N2; ExpRF4
N2; ExR13H8(1Fer)
N2; ExR13H8(2Fer)
N2; ExR13H8(1Str)
N2; ExR13H8(2Str)
age-1(hx546); ExpRF4

Fig. 2A

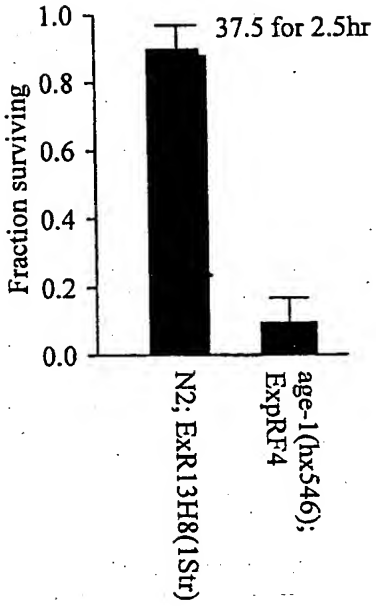
37.5 for 2.5hr
Fraction surviving
1.0
0.8
0.6
0.4
0.2
0.0
N2; ExR13H8(1Str)
age-1(hx546); ExpRF4

Fig. 2B

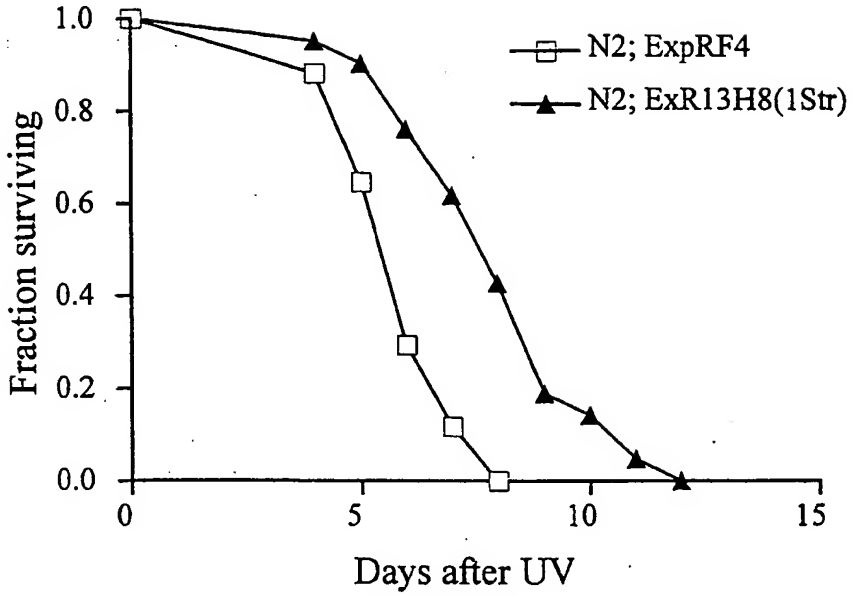
1.0
0.8
0.6
0.4
0.2
0.0
Fraction surviving
0
5
10
15
Days after UV
N2; ExpRF4
N2; ExR13H8(1Str)

Fig. 2C

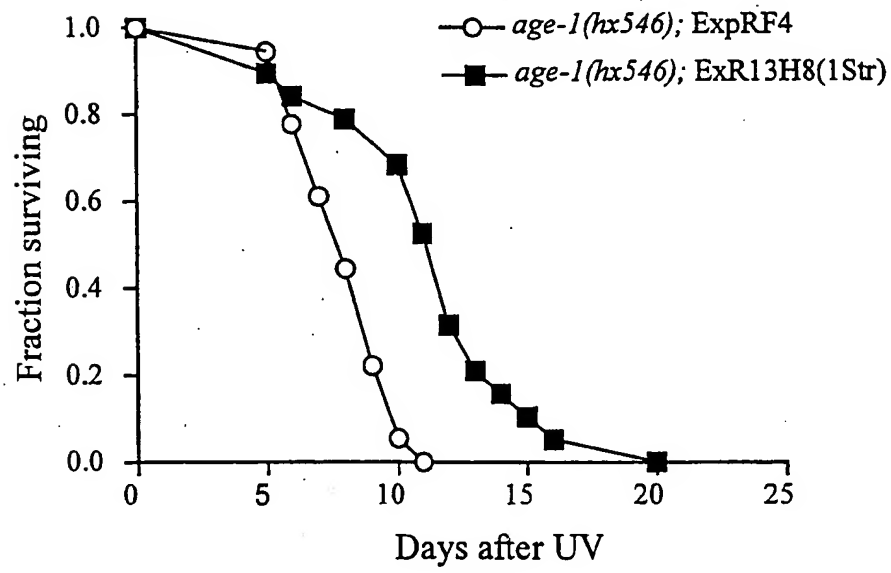
1.0
0.8
0.6
0.4
0.2
0.0
Fraction surviving
0
5
10
15
20
25
Days after UV
age-1(hx546); ExpRF4
age-1(hx546); ExR13H8(1Str)

Fig. 2D

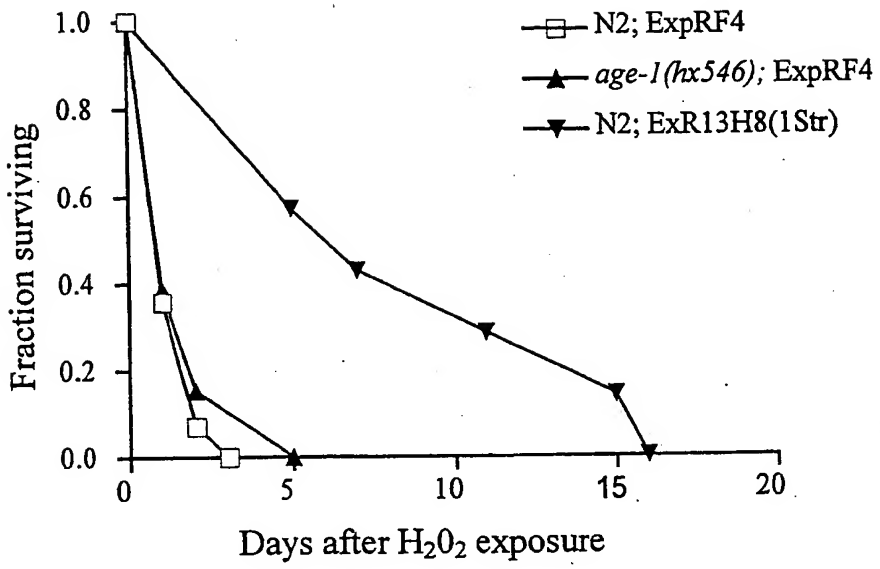
N2; ExpRF4
age-1(hx546); ExpRF4
N2; ExR13H8(1Str)
Fraction surviving
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
Days after H_2O_2 exposure

Fig. 2E

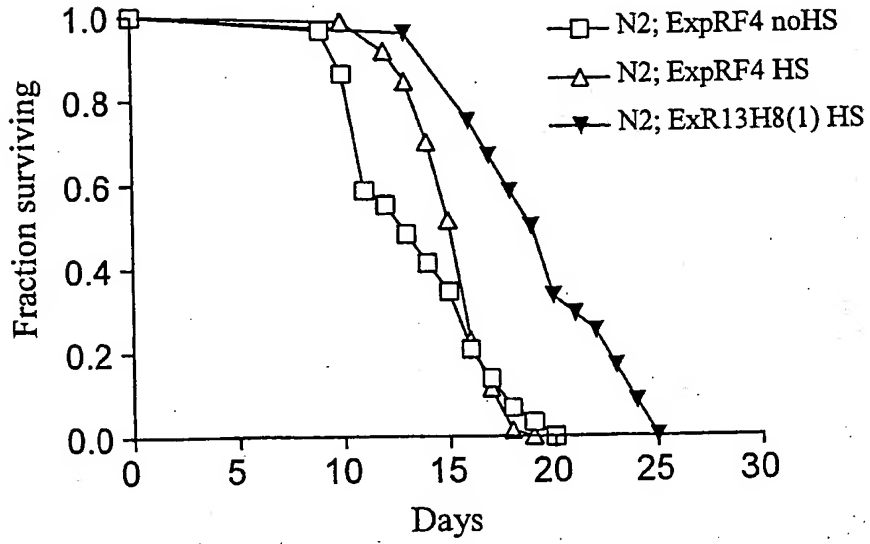
N2; ExpRF4 noHS
N2; ExpRF4 HS
N2; ExR13H8(1) HS
Fraction surviving
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Days

Fig. 3

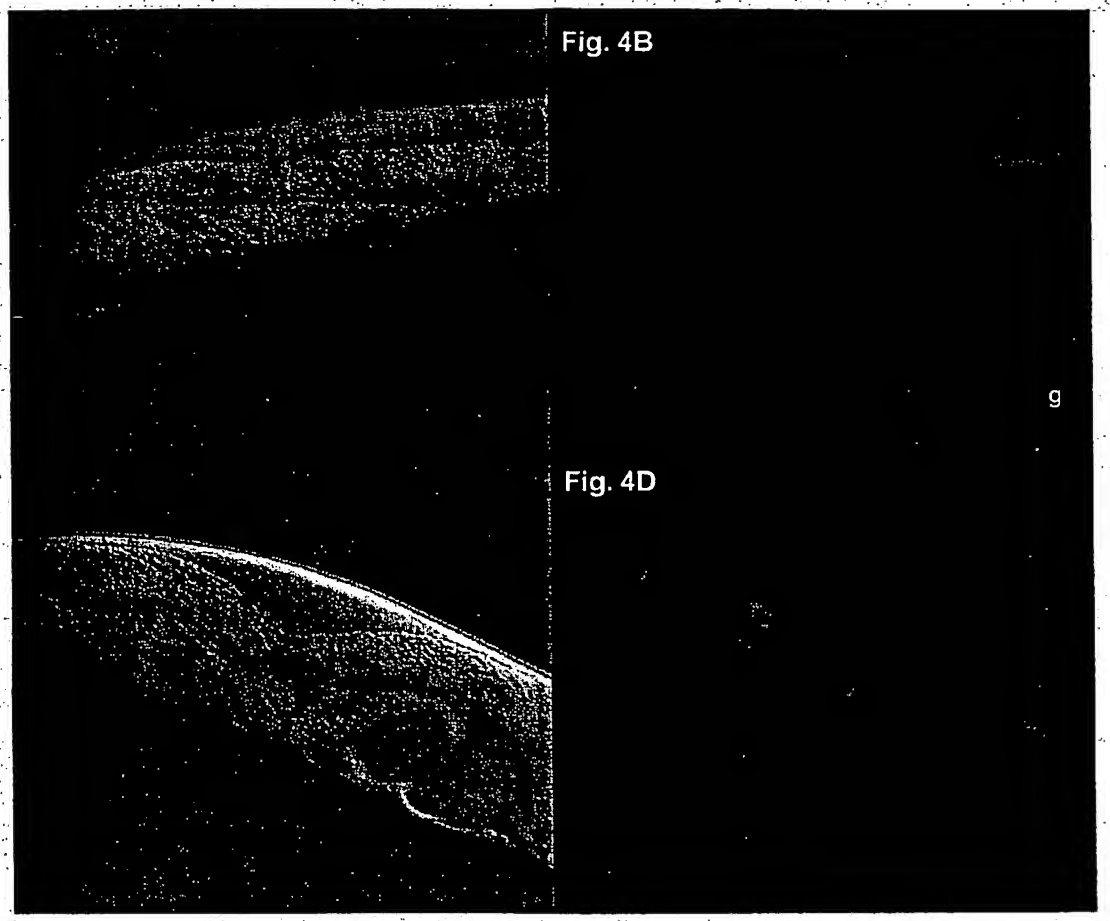
Fig. 4B
Fig. 4D
g

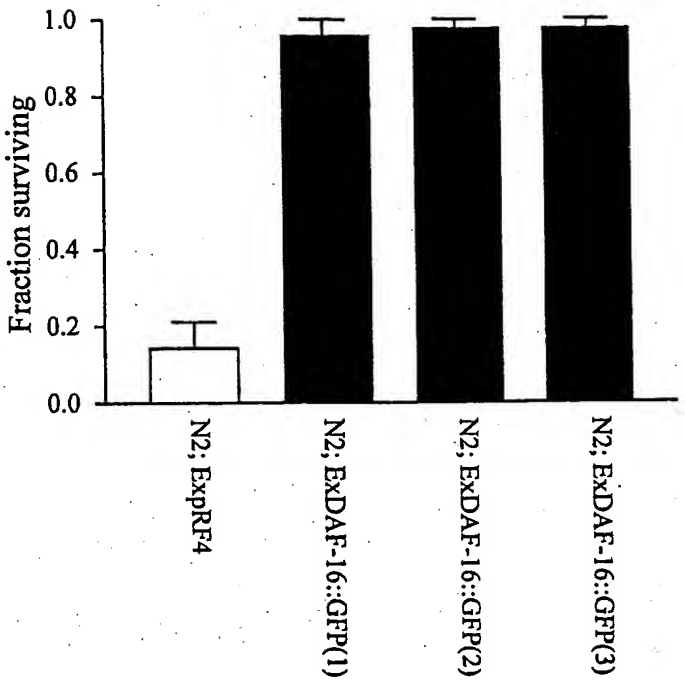
Fraction surviving
1.0
0.8
0.6
0.4
0.2
0.0
N2; ExpRF4
N2; ExDAF-16::GFP(1)
N2; ExDAF-16::GFP(2)
N2; ExDAF-16::GFP(3)

Fig. 5A

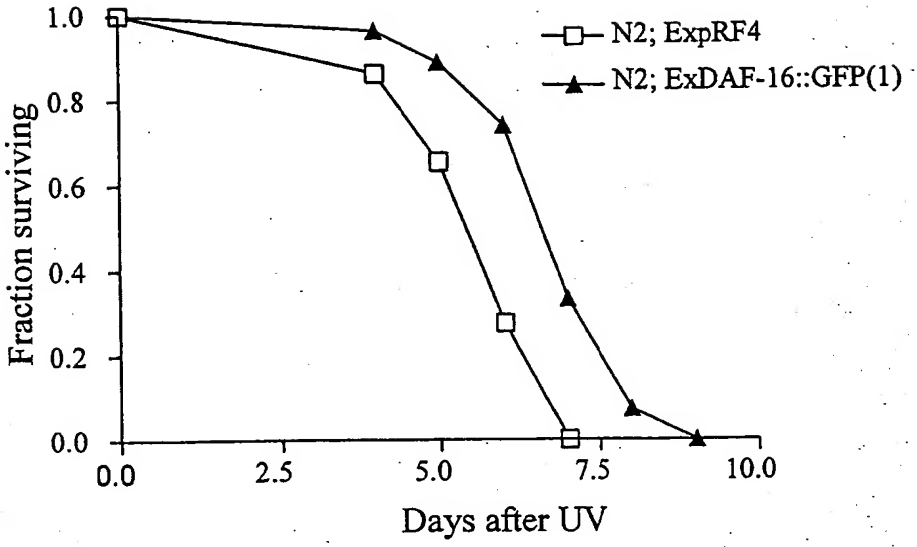
Fraction surviving
1.0
0.8
0.6
0.4
0.2
0.0
0.0
2.5
5.0
7.5
10.0
Days after UV
N2; ExpRF4
N2; ExDAF-16::GFP(1)

Fig.5B

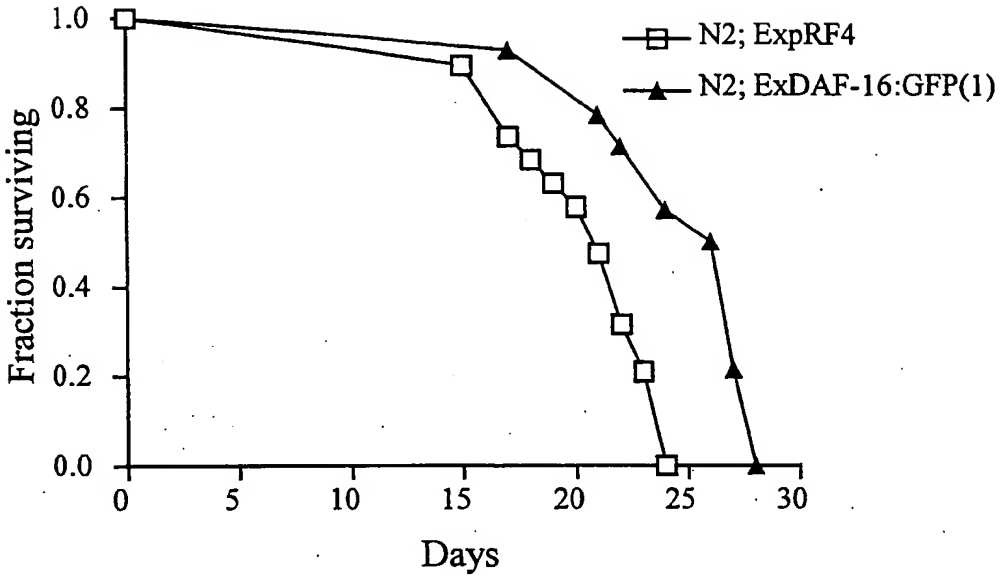
1.0
0.8
0.6
0.4
0.2
0.0
Fraction surviving
0
5
10
15
20
25
30
Days
N2; ExpRF4
N2; ExDAF-16:GFP(1)

Fig. 5C

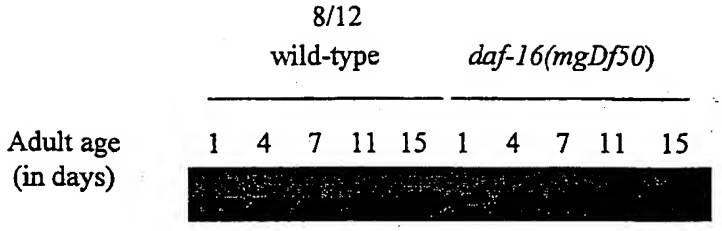

Fig. 6A

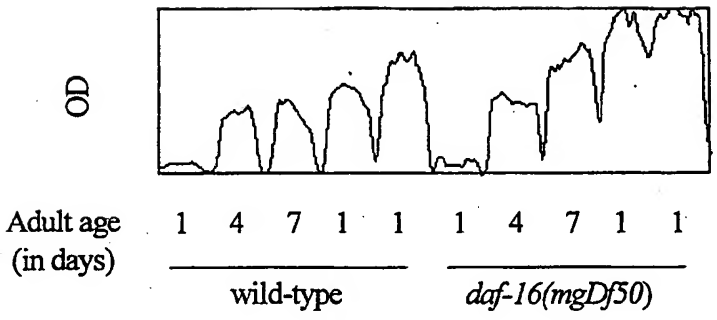

Fig. 6B

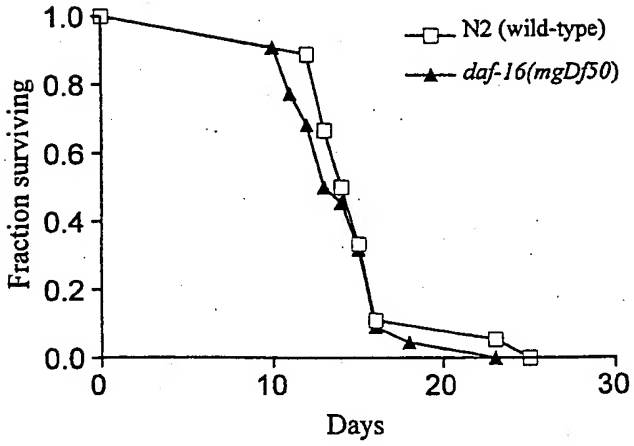

Fig. 6C

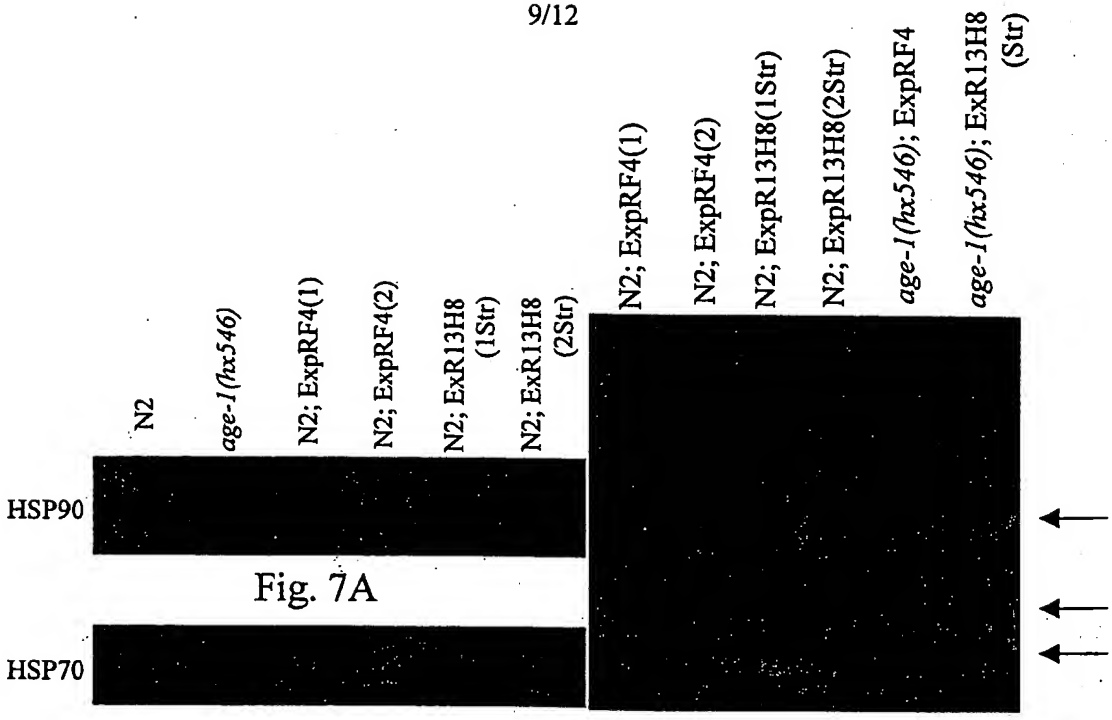

Fig. 7A

Fig. 7B

Fig. 7C

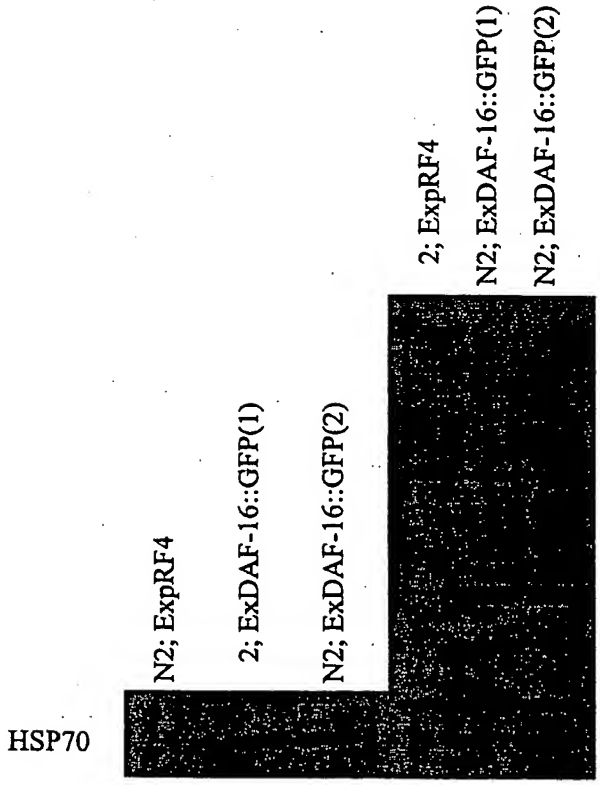

Fig. 7D

Fig. 7E

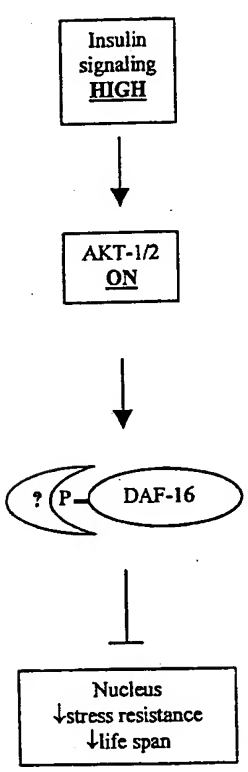

Fig. 8A

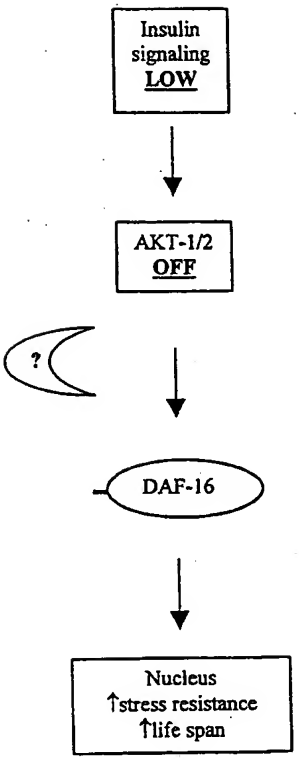

Fig. 8B

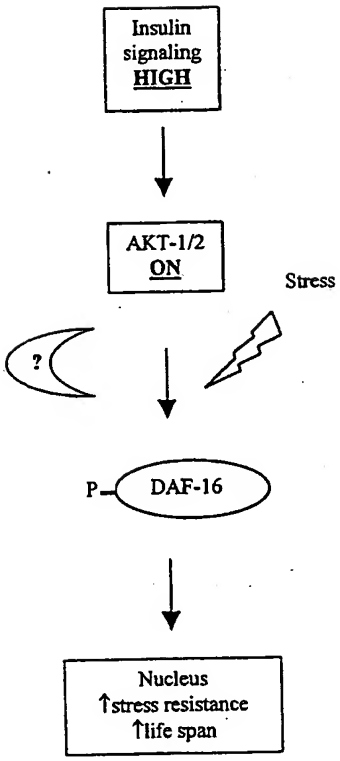

Fig. 8C

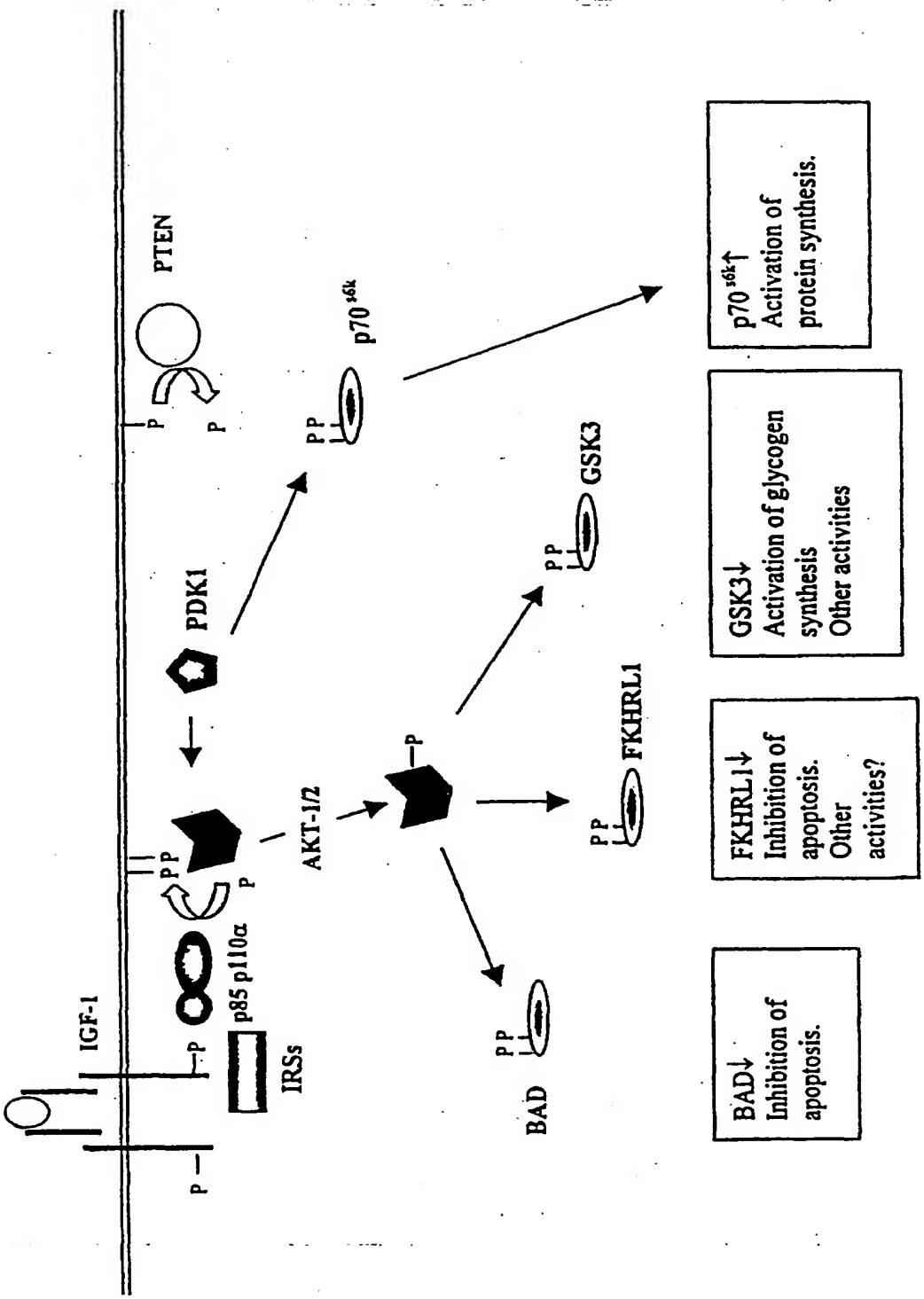
IGF-1
IRSs
p85 p110α
PTEN
PDK1
AKT-1/2
p70 s6k
GSK3
FKHRL1
BAD
p70 s6k↑ Activation of protein synthesis.
GSK3↓ Activation of glycogen synthesis Other activities
FKHRL1↓ Inhibition of apoptosis. Other activities?
BAD↓ Inhibition of apoptosis.

Fig. 9

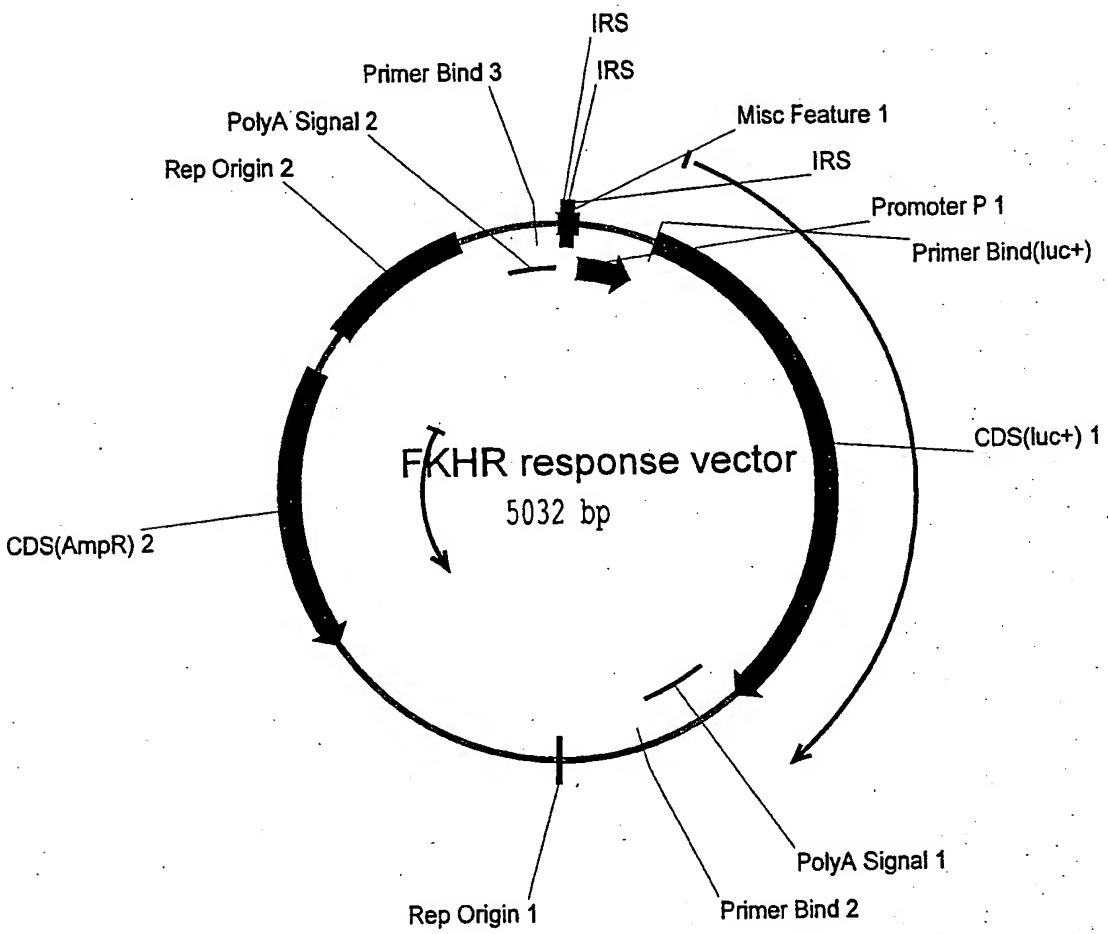
IRS
IRS
Primer Bind 3
PolyA Signal 2
Misc Feature 1
IRS
Rep Origin 2
Promoter P 1
Primer Bind(luc+)
FKHR response vector
5032 bp
CDS(luc+) 1
CDS(AmpR) 2
PolyA Signal 1
Rep Origin 1
Primer Bind 2

Fig. 10

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/24487

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 51351 A (GEN HOSPITAL CORP) 19 November 1998 (1998-11-19) cited in the application	1-51
Y	the whole document	1,5,6, 10,16, 20,28, 32,47,51
Y	RENA G ET AL: "Phosphorylation of the transcription factor forkhead family member FKHR b protein kinase B." JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 JUN 11) 274 (24) 17179-83. , XP000981982 the whole document	1,5,6, 10,16, 20,28, 32,47,51

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 February 2001	22/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gundlach, B

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/24487

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KOPS G J ET AL: "Direct control of the Forkhead transcription factor AFX by protein kinase B." NATURE, (1999 APR 15) 398 (6728) 630-4. , XP000955388 the whole document ----	1,5,6, 10,16, 20,28, 32,47,51

Form PCT/ISA/210 (continuation of second sheet) (July 1992)